METHODS IN MEDICAL RESEARCH

Volume 7 · J. V. WARREN, *Editor-in-Chief*

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METHODS IN

MEDICAL RESEARCH

Volume 7

METHODS IN

Medical Research

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Volume 7

JAMES V. WARREN, *Editor-in-Chief*

CHEMICAL INVESTIGATION OF MUSCULAR TISSUES,
W. F. H. M. Mommaerts, Editor

HEMODYNAMIC METHODS—HEART AND LUNGS, *Richard Gorlin and James V. Warren, Editors*

METHODS FOR THE STUDY OF HUMAN LEUKOCYTES, *Samuel P. Martin, Editor*

METHODS FOR STUDY OF THE HISTOLOGY AND CYTOLOGY OF THE RETINA,
E. N. Willmer, Editor

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GOVERNING BOARD PREFACE

When this series of annual volumes was begun the Governing Board had misgivings of two general types. One had to do with the long-range usefulness and popularity of the series, the other with the willingness of large numbers of busy colleagues to dedicate the time and energy required to carry out the original plan of multiple authorship and free cross-criticism. As noted in the prefaces to recent volumes, these misgivings have proved unfounded. Since the readers can scarcely have gotten out of the books more than the authors put into them, the continued popularity of the series must be construed as a tribute to the intelligence and industry of the various editors and their associates. The Governing Board wishes to take this opportunity to express its own appreciation, undoubtedly shared by the many scientists who will find this volume useful, to Dr. Warren and his collaborators in the preparation of Volume 7.

For the first time in the series, however, it becomes necessary to acknowledge a set of delays which could not be obviated even by the best efforts of all concerned. In view of the large amount of interdigitation and articulation involved in each of these annual enterprises, this was bound to happen sooner or later. We can only hope that the misfortunes which beset the preparation of Volume 7 will not soon recur and that the good luck of its predecessors will return to make possible the publication of subsequent volumes on the usual schedule.

The Governing Board originally expected to reschedule some previously covered topics after an interval of about 10 years. As this time approaches, the Board will be even more grateful than usual for suggestions as to topics for future volumes.

Irvine H. Page
René J. Dubos
C. N. H. Long
Carl F. Schmidt
David L. Thomson

EDITOR'S PREFACE

An Editor for a volume of Methods in Medical Research is inclined to ponder on the direction that medical thought and research is taking. He must consider what kind of methodologic help is needed. Currently much attention in scientific fields is devoted to the study of the larger units of the universe. Light years and satellites are the working units. Interestingly enough, in medical research thoughts are turning more and more in the direction of smaller units. The microscope was only the beginning of this trend. Functions of the cell are being examined in terms of its chemical structure and activity in terms of molecular constellations. Medical research in particular reflects this tendency, and the present volume is no exception. The tide is clearly running in the direction of an understanding of the activities of the single living cell, explained in chemical terms rather than in gross morphologic activity of an organ.

The section edited by Dr. Mommaerts specifically demonstrates the tendency toward attempts at basic understanding of biochemical activity of cellular groups. The methods described are those utilized in explaining why the muscle contracts rather than what are the gross physical phenomena associated with its contraction. The section edited by Dr. Martin utilizes the human leukocyte as an interesting microcosm. With these methods one studies cytologic, immunologic and biochemical factors in a preparation of a single cell type. The section edited by Professor Willmer, although apparently utilizing more conventional technics in studying the histology and cytology of the retina, shows that here, too, our interests have gone beyond morphology and are also aimed at probing cellular activity. The cardiovascular methodology edited by Drs. Gorlin and Warren may appear to be a return to the traditional. In part this is true, but it is of interest to note that the technics described here have in recent years been applied with increasing frequency to the study of the chemical activity of the vascular tissues. The method of determining coronary blood flow, for instance, has been primarily utilized to study myocardial metabolism. It should be pointed out that this section represents in effect an appendix to the section on related methodology which appeared in Volume 1 of this series.

It has been noted in prefaces of earlier volumes of these scientific cookbooks that the role of the editor is a relatively small one. The

associate editors and their contributors really provide the content of these volumes, and it is to them that credit must be given. The birth pains of the present volume have been considerable. Delivery has been made possible only through the encouragement of members of the Governing Board and the staff of The Year Book Publishers. To both groups we owe a great debt of gratitude.

James V. Warren

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SECTION I

Chemical Investigation of Muscular Tissues

ASSOCIATE EDITOR—*W. F. H. M. Mommaerts*

INTRODUCTION

It is not the purpose of this section to give an exhaustive account of all chemical observations which can be made on muscular tissues; many methods which, at one time or another, have been applied, are of too limited significance to need reviewing. Others are of general importance and not specifically linked with myochemistry. A complete listing of all such procedures would approach the size of a handbook of biochemical analysis. Instead, it is only intended to give an arbitrary selection of procedures with no other criterion than that they seem to be related to certain recent trends in the investigation of contractile activity. There seems to be a need for such a presentation, since observations on myosin, actin, nucleotides, phosphate metabolism and the like begin to attract a wide interest not only from the side of pure biochemistry and physiology, but also in relation to problems in pathology and experimental medicine.

The available material could be divided in two broad sections, comprising methods of preparation and analysis, and methods of measurement and experimentation. The distinction is, of course, not sharp, and will not be belabored unnecessarily. It implies only that in the first group of procedures (parts A and B), attention would be focused on single substances, their preparation, detection and analytical determination; while the second group would comprise more complicated experimental procedures, including notably certain physicochemical measurements and some types of experi-

ments on complex systems. Only a few examples belonging to the latter category will be given (part C).

In each instance, complete and exhaustive prescriptions will be attempted; indeed, with few exceptions, all methods are or have been used in my laboratory.

The choice of examples will reflect the fact that, among different contractile tissues, most work is still being done with skeletal muscle; cardiac muscle is studied less extensively; while smooth muscle is as yet rather neglected, which is regrettable in view of the considerable physiologic and pharmacologic interest which attaches to this quite heterogeneous category of tissues. Within each group of tissues, the choice of representatives has also been narrow. Thus, glycolytic enzymes and the structure proteins (actin and myosin) are usually prepared from the leg and back muscles of domestic rabbits; respiratory granules are mostly made from mammalian hearts or from pigeon breast muscle; for some types of experiments on metabolism and physiologic activity, frog muscles have been the subject of choice. Such limited variation is common practice in science, since the penetrating study of a few favorable materials is preferable to a superficial survey of a large variety of subjects. When new types of problems arise, none of the hitherto favored materials may permit a successful approach. For example, the gastrocnemius and other leg muscles of the frog have been very suitable for study of the metabolism associated with shorter or longer periods of tetanic activity, as well as for physical (e.g., thermometric) measurements during a single twitch; but for investigation of some of the chemical changes during a single twitch this type of muscle is too fast, and recourse must be had to the slower skeletal muscles of turtles. Alternatively, one could choose the papillary muscle of the cat's heart, or still more slowly acting smooth muscle. The disadvantage in these cases is that there is much less general chemical knowledge available for these categories of tissue with which to compare or correlate the obtained results. Also, whenever one muscle has to be sacrificed for the analysis, the contralateral member of a symmetric pair is necessary as the control; this further limits the use of the papillary muscle preparation.

As in other fields, therefore, the careful choice of a suitable experimental object is a most essential step in planning a new investigation.

W. F. H. M. Mommaerts.

I. PREPARATION OF STRUCTURE PROTEINS AND OF ENZYMES FROM RABBIT SKELETAL MUSCLE

DISSECTION AND EXTRACTION OF RABBIT MUSCLE

A large number of proteins and enzymes has been prepared from this material, which has, among others, the advantage of easy availability. In general, female white rabbits of about 5 lb weight are preferable. They should be fed only moderately, and a day's starvation before use is recommended; when working in hot climates we often kept the animals in a refrigerated room during that day. For many purposes, the muscles of other animals, such as dogs or rats, can be substituted, but less experience is available in those instances. Invariably, the material must be obtained in a resting, flaccid condition, subjecting it to a minimum of stimulation. This can be achieved by several means. Often it suffices to kill the animal by a blow on the head or by bleeding from the neck, but much depends then on the dexterity of the investigator; it is better to resort to some form of anesthesia. The common anesthetics, such as Nembutal, are often used, and wide use has been made of Meltzer's narcosis, $MgSO_4$. A 25% solution of this (1–2 ml/kg body weight) can be injected intravenously or intraperitoneally; in the latter case the results are more variable and the dose may have to be repeated. $MgSO_4$ has often been used for the preparation of ATP (1) and of myosin (2); its beneficial influence in these cases is certainly quite unrelated to its inhibitory effect upon myosin adenosinetriphosphatase, but this is an academic question only.

Recently, Myanesin (mephenesin) has been used to obtain relaxed muscles; the dose is stated to be 300 mg (3) or 600 mg (4) per kg, but we find that even with this high dose complete anesthesia is not regularly obtained on intraperitoneal injection; regular use of large doses is also very expensive. It is recommended, however, that this drug be used before other treatment because it acts as a central depressant.

Curare is a most effective relaxant, of course. It is now available in pure preparations of reproducible activity, e.g., tubocurarine, of which 0.25–0.5 mg per kg injected intramuscularly into the foreleg is effective within 5–15 min. Since curare does not depress sensation, the animal must be rapidly killed by quick exposure and section of the carotid artery; the procedure may be combined with previous application of a moderate dose of Myanesin.

After the animal is exsanguinated, the skin is rapidly incised behind the shoulders around the body, and stripped off, after which the viscera are removed. Usually, only the hindpart of the body, behind the anterior attachment of the psoas muscle, is used. Some recommend that the muscles be excised at once and packed in ice, but it is more usual first to cool the entire carcass. According to Dubuisson (5, 6), this should not be done by direct immersion in ice water but gradually by exposure to the air in a refrigerator, since sudden contact with cold media causes some stimulation. However, if curare is first injected, the use of ice probably has no such adverse effects.

After the muscle has cooled in ice for some 15 min it is dissected and ground. Relatively crude mincing is sufficient and preferable for most purposes. A hand-operated meat grinder of good design and heavy construction, with an endplate with 3–4 mm holes and with knives rotating against the plate, is suitable. For larger quantities, a motor-driven grinder, such as used in butcher shops, is more practical; they can now be obtained with stainless steel grinding gear. Sometimes much finer disintegration is required, and can be done by means of a Waring Blendor. Most muscles, such as the combined musculature of rabbits, contain too much connective tissue to be homogenized in a Blendor without previous mincing; only the rabbit psoas muscle can be so treated. For special purposes, use of a Latapie mill (after the muscle has been ground and thorough removal of connective tissue), or a freezing microtome, or of specially designed equipment may be advantageous. The meat is kept cold during the operations, at least to the extent of storing it in ice and of collecting it in an ice-cooled receptacle or directly into ice-cold extractant immediately after grinding.

Extraction with Water

Many proteins and enzymes are removed from muscle by extraction with water which, diluting the electrolytes contained in the muscle's intra- and extracellular fluids, corresponds to extraction with a solution of an ionic strength of 0.1 or below. Proteins so extracted are collectively called *myogen* (including, in this case, the so-called globulin X which precipitates upon exhaustive dialysis against water). Myogen consists largely or wholly of enzymes, and is the usual starting material for the preparation of glycolytic enzymes. For some of these, and for the extraction of myogen as such (e.g., for electrophoretic analysis), special extractants have been recommended, buffered solutions of relatively low ionic strength. The modern tendency, originated by the Cori's, and recently fur-

ther developed in Bücher's laboratory, is to perform the original extraction in a uniform manner, and to design the fractionation so that several enzymes are obtained subsequently from the single batch of extract. The initial extraction according to Cori is done by stirring for 30 min in the cold with 2 vol of 0.03*N* NaOH, then draining the fluid off through several layers of gauze tied as a bag over a glass jar or placed in a porcelain funnel, and finally re-extracting the residue with 1 vol of water or KOH. In the Bücher procedures, the extractant is water containing 0.5 g of Versene per liter, the operations otherwise being identical.

Of the many proteins and enzymes present in muscle, we shall mainly deal with the structure proteins and with those enzymes which are important as analytic tools.

GENERAL REMARKS ON MUSCLE ENZYME FRACTIONATION

Most muscle proteins are exceedingly labile and are easily damaged by various denaturing influences. In part, their inactivation may be caused by oxidation of sensitive sulfhydryl groups catalyzed by traces of heavy metal, and several precautions are necessary on this account. All water used must be redistilled from an all-glass still, or repurified by filtration through a frequently renewed mixed-bed ion exchange resin tower (e.g., a so-called water demineralizer of all-plastic construction). Glassware that becomes contaminated with heavy metals (e.g., pipets used for delivering Nessler reagent) must be soaked in Versene solution before final cleaning. Metal tools used in the preparation, such as meat grinders, must be kept spotlessly clean. It is also recommended that Versene be added during the extraction of the tissue, but there is as yet insufficient experience on this point, except in the preparation of glycolytic enzymes according to Bücher *et al.*

Ammonium Sulfate

Fractionation by salting-out with ammonium sulfate is one of the most important elements of several fractionation schemes. Crude extracts can be fractionated with this salt as such, or in the presence of Versene. For later stages of fractionation, it is inadvisable to add sequestering agents, since they may be carried over into the final system and cause difficulty by binding Mg or other essential ions. It is then necessary to use ammonium sulfate recrystallized from a hot saturated solution after adding ammonia to pH 8 and 2 g Versene per liter (29). A saturated ammonium sulfate solution is prepared by stirring 1 liter of water at room temperature with about 800 g of the salt, or, for a cold saturated solu-

tion, with about 720 g of the salt, followed by cooling in the cold room, and storing it with a small excess of solid at the respective temperature. At 0°, a saturated solution is about 3.9M, at 20° it is 4.1M; its pH is about 5.2. A neutralized saturated ammonium sulfate solution is prepared by adding a specified amount of concentrated ammonia to obtain the desired pH.

Ammonium sulfate precipitates can be collected by strong centrifugation or by filtration by suction or under pressure. The choice largely depends on the available facilities in terms of economy of time. Filtration can be facilitated with filter aids such as Hyflo Supercel, and is always performed when the solution rather than the precipitate is to be used in the further course. It is inconvenient when the precipitate is to be worked up, and when, as in procedures utilizing the so-called 52-72 paste, the method is based upon assuming a certain ammonium sulfate concentration in the wet paste.

In older procedures, the ammonium sulfate concentration is mostly expressed in terms of its percentual or fractional saturation (e.g., 0.72 sat) at a specified temperature. (Unfortunately, complete specifications of the temperature and pH are usually lacking in the literature.) It is more accurate, however, to express the concentration in molarity (29). When it is necessary to increase the salt concentration stepwise, it is best achieved by slowly adding the calculated amount of dry salt while stirring. The following table (29) gives the relation between the molarity, M, and the percentage (g salt/100 g solution), g:

M	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9	1.0
g	1.3	2.6	3.8	5.1	6.4	7.6	8.8	10.0	11.1	12.3
M	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	2.0
g	13.4	14.6	15.7	16.8	17.9	19.0	20.1	21.2	22.2	23.3
M	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9	3.0
g	24.3	25.3	26.4	27.4	28.4	29.4	30.3	31.3	32.2	33.2
M	3.1	3.2	3.3	3.4	3.5	3.6	3.7	3.8	3.9	
g	34.2	35.1	36.1	37.0	38.0	38.9	39.8	40.7	41.6	

The amount of salt, X g, required to bring V ml of solution of molarity M_1 (percentage P_1) to percentage P_2 (molarity M_2) is:

$$X = V\left[\frac{P_2(100 - P_1)}{P_1(100 - P_2)} - 1\right]\frac{M_1}{7.6}$$

In older procedures, the change in concentration is usually brought about by adding specified volumes of saturated ammonium sulfate; several such examples will also be given.

Calcium Phosphate

Calcium phosphate gel for the adsorption of enzymes or of contaminating proteins is prepared according to Keilin and Hartree (7). Prepare stock solutions of A (132 g $CaCl_2 \cdot 6H_2O$ per liter), and B (152 g $Na_2PO_4 \cdot 12H_2O$ per liter). Add 150 ml of solution A to 1,600 ml of water, shake this with 150 ml of solution B, and bring the pH to 7.4 with dilute acetic acid. The precipitate is washed 4 times with 15 liters of water by decantation, then 2 times with a smaller volume of water by centrifugation. The yield is about 9 g which are suspended in water to a final concentration of about 1 g in 40 ml. The suspension is aged for several months before use.

Alumina gel Cγ

This is among the most useful adsorbents and is prepared, with some modifications introduced by G. T. Cori, according to Willstätter *et al.* (8). Prepare a solution by adding 205 g ammonium sulfate and 316 ml concentrated ammonia to 6.6 liters distilled water heated to 55° C, and filter if necessary. To this, a solution of 520 g $(NH_4)_2Al_2(SO_4)_4 \cdot 24H_2O$ in 1 liter of hot water is added slowly, with constant vigorous stirring; the mixture is then stirred for 15 min at about 60° C and is then allowed to cool. By sedimentation, decantation and siphoning the precipitate is washed 10 to 15 times with about 5 liters of water, until (as indicated by an opalescence of the supernatant solution) contaminating ions have been removed. After 3 to 5 more washings, the suspension is left at room temperature for 1 to 2 weeks; the supernatant solution is siphoned off completely, and the suspension (which usually contains 20 to 25 mg of alumina gel per ml and is ready for use) is stored in the cold.

PREPARATION OF STRUCTURE PROTEINS

EXTRACTION AND PRELIMINARY PURIFICATION OF MYOSIN AND ACTOMYOSIN

Both myosin and actomyosin are prepared by extracting minced rabbit muscle in the cold with strong salt solutions, such as 0.5M KCl, or buffered KCl solutions with an ionic strength of about 0.5. Apart from the soluble proteins of the myogen group, these extracts contain a mixture of myosin and actomyosin. The mixture arises because the salt solution, in the presence of ATP, dissolves myosin primarily, but also detaches actin or actomyosin from the surfaces of the muscle fragments; more actin is released in the course of time when the ATP present is decomposed. Thus, pro-

longed extraction, fine mincing, and stirring during the extraction all promote the predominance of actomyosin in the mixture. The composition of the extractant also has an effect. A fairly pure myosin is obtained by extracting coarsely minced muscle for 15 min with 0.4M KCl, 0.05M K_2HPO_4 and 0.10M KH_2PO_4 (Guba-Straub solution), whereas a fairly pure actomyosin is obtained by prolonged extraction (24 hr) with stirring with Guba-Straub solution, with 0.5M KCl, 0.03M $NaHCO_3$, or with 0.6M KCl, 0.04M $NaHCO_3$, 0.01M Na_2CO_3 (Weber-Edsall solution). The exact composition of these solvents is not critical and can be modified for special purposes.

From such extracts, myosin and its complex can be precipitated by reducing the ionic strength to about $^1/_5$–$^1/_{12}$ of its original value. At ionic strengths above 0.1, precipitation of myosin may not be complete, especially at alkaline reaction. This precipitation is usually achieved by diluting the extract with 5–12 vol of water, but rapid dialysis has also been applied (2); attempts to remove the salt with a mixed cation-anion exchange resin were not successful due to denaturation of the protein, although the pH remained within a suitable range. After collecting the precipitated protein by centrifugation (frequently it settles by gravity in 2–3 hr, reducing the otherwise large volume to be centrifuged), it is dissolved by adding a 2M KCl solution to the voluminous precipitate to a final concentration of about 0.5M. The precipitation can then be repeated; in this manner, the bulk of the contaminating proteins of the myogen group can be removed in 2 or 3 operations, and the resulting myosin–actomyosin mixture is suitable for teaching and demonstration purposes, and for orientating investigations.

PREPARATION OF CRYSTALLINE MYOSIN

The purification of myosin was achieved in the following stages. Szent-Györgyi (11) obtained a crystalline myosin free of actin, but still, as was shown later, contaminated with denaturation products; Weber *et al.* (1949) obtained a pure, not denatured but not crystallized preparation, and Mommaerts and Parrish (1950) achieved crystallization of the pure product without denaturation. The method here given combines features of all three procedures, and is essentially in the form given by Green *et al.* (9).

Coarsely minced rabbit muscle, 300 g, is extracted in the cold with 1 liter of 0.3M KCl, 0.10M KH_2PO_4 and 0.05M K_2HPO_4, for 15 min with gentle stirring (rather extensive trials during 1951–1952 showed no effect from the addition of Versene to the extractant, except for somewhat higher yields); 4 liters of cold water are added to the mixture, which is at once strained through several

layers of gauze. The filtrate is further diluted with water to 12 liters, and left in the cold for 3 hr. The supernatant solution is siphoned off as far as practicable, and the protein is collected by centrifugation. The precipitate is dissolved by the addition of 60 ml of 2M KCl and 30 ml of 0.25M KH_2PO_4 and 0.25M K_2HPO_4, and the solution is made up with water to 300 ml (ionic strength 0.5; pH about 6.7). This solution is clarified by centrifugation and by filtration through a coarse sintered-glass filter; the latter manipulation serves to remove floating fat particles. The solution is now diluted to an ionic strength of about 0.28 by the addition of 240–250 ml of water, causing actomyosin to precipitate (10). This is removed by strong centrifugation, e.g., 30 min at 10,000 rpm. The supernatant solution is placed in a cooled vessel, and 4 liters of cold water are slowly added (over a period of about 10 min) with continuous stirring with a plastic or glass spade-shaped stirring blade. The myosin will separate in the form of fine crystalloid needles (11) which are visible in the microscope and which lend a silky appearance to the flowing solution. The protein is collected by centrifugation and is dissolved in KCl solution with added buffers as desired. There are no systematic studies on the effects of individual buffer mixtures upon the stability of the protein. If necessary, the crystallization step can be repeated, or an amorphous precipitation (by adding the same proportion of water suddenly without stirring) can be introduced before the crystallization. The protein so obtained is of high purity according to several criteria (2, 10).

PREPARATION OF ACTOMYOSIN

The initial extraction of the muscle is the same as for the preparation of myosin, but the extraction lasts for about 20–24 hr in the cold with gentle stirring. A somewhat larger proportion of solvent to tissue can be taken, e.g., 5 vol. The mixture will now be so viscous that upon centrifugation the swollen muscle residue will settle with difficulty; this can be improved somewhat by adding, immediately before centrifugation, more Guba-Straub solution containing about 50 mg of ATP for each 100 g of muscle. A better procedure is to employ high speed centrifugation, e.g., 30 to 60 min at 10,000–15,000 rpm. The supernatant solution is poured off and filtered through some layers of gauze or glass wool, or through a coarse sintered-glass filter.

This crude extract, too, contains the soluble muscle proteins in addition to those of the myosin group, and variable amounts of uncombined myosin besides actomyosin. The former can be removed by repeated precipitation of the myosins at low salt concentration,

the latter by precipitating the actomyosin at ionic strength 0.28, pH 6.7 (10), as described in the purification of myosin. These steps can be alternated and combined in various ways.

Since actomyosin seems to retain contaminating enzymes, such as myokinase, rather tenaciously, we have adopted the following procedure in order to minimize this complication (12).

The muscle extract (400 ml) is poured into 5 vol of cold water, stirred gently for a few minutes, and the protein is centrifuged down. The sediment is taken up in 300 ml cold water and stirred (without whipping) until the protein is well suspended. The extract is then added to 300 ml water of 25–30° C, containing 25 mg of neutralized ATP. After 5 min of stirring, the suspension is cooled in a cold bath, and centrifuged. The precipitate is dissolved with 50 ml of cold 2M KCl and made up with water to 200 ml; the pH is adjusted to 6.7–6.8, the extract is diluted with 160 ml of water, and the actomyosin is collected by strong centrifugation. The sediment is suspended in 500 ml of water, centrifuged off again, and dissolved with 50 ml of 2M KCl to a final volume of 200 ml. Buffers can be added according to the requirements. Actomyosin so prepared is free of demonstrable amounts of myokinase.

PREPARATION OF CRUDE ACTIN

The starting point of the preparation is the muscle residue left after the extraction of myosin. Actin can also be obtained from the residue after the extraction of water soluble enzymes and even after the extraction of adenylate deaminase (p. 14); the yield may be lower in that case, but it is economical for laboratories where large amounts of tissue are used for enzyme preparations.

The first step is the preparation of an acetone powder after the method of Straub (13), discoverer of actin, to which some modifications have recently been proposed (14).

The muscle is extracted for myosin during 15 min, the mixture diluted with water as described, and filtered through gauze. The residue is washed in the cold by gentle stirring in 4 vol of 0.05M $NaHCO_3$ (a pH of about 7 results) for 20 min, strained through gauze, resuspended in 10 vol of water, and stirred for 20–30 min. It is again filtered through gauze and pressed by hand as dry as possible. The residue is now extracted twice with 2 vol of ice-cold *n*-butanol and twice with 2 vol of acetone at room temperature; each time, the mixture is briefly disintegrated in a Waring Blendor, then left suspended for 15–30 min, and filtered through gauze and pressed as dry as possible. After the last extraction, the material is spread out on a glass tray and left to dry in the air. It can be stored

in a jar over silica gel, but even at low temperature the material usually deteriorates within a few weeks.

In order to extract actin, the dry mass is suspended in 25 vol of cold ATP solution (60 mg/liter, pH 8.2); the material, after butanol-acetone treatment, does not wet easily, and must be stirred or kneaded to assure wetting. After 30 min extraction in the cold, the mixture is poured into a Büchner funnel and filtered by suction; the residue may be re-extracted with about half of the originally used volume of ATP solution. The solution so prepared contains globular or G-actin, usually of about 30–50% purity and about 5 mg of protein per ml but the results are quite unpredictable.

PREPARATION OF PURE ACTIN (15)

The purification of actin is not based on the traditional sequence of precipitation or adsorption procedures, but on a highly specific property of this protein: the ability to be transformed into a polymer, F-actin, by the addition of neutral salt. The polymerized protein is separated from the unpolymerized impurities by preparative ultracentrifugation, and is reconverted into the monomer.

The method starts with the preparation of an acetone-dried muscle powder, as described for crude actin; 50 g of powder are extracted for 30 min in the cold, with gentle stirring, with 1 liter of 2×10^{-4}M ATP solution at pH 8.0–8.2, which solution has first been freed of air with a current of nitrogen or helium; anaerobic conditions are maintained during the extraction as far as feasible. The suspension is then filtered through paper by suction, and is re-extracted with 300 ml of water or ATP solution. The combined extracts, 600–900 ml, are clarified by centrifugation for 1 hr at about 10,000 rpm.; 20 ml of 2M KCl solution are added to the extract, and polymerization is allowed to proceed for several hours at room temperature. The progress of polymerization is best tested by observing the flow birefringence of a sample of the solution placed in a beaker glass, swirled between crossed polaroids; an even simpler way is to watch the increase in viscosity, which is so obvious that it can be seen from the slow rotation of the fluid set in motion by swirling, and from the slow rise of air bubbles trapped by the moving solution.

After polymerization, the actin is collected by centrifugation in a No. 30 rotor of a Spinco preparative ultracentrifuge, for 2 hr at 30,000 rpm; the extract is centrifuged in two batches, the second sample being sedimented on top of the pellets from the first run. The pellets are dissolved in the cold in 400 ml of 2×10^{-4}M ATP

solution at pH 8.2, by rapid stirring without foaming for 2 hr; 10 ml of 2M KCl is added to reverse any depolymerization during the dissolution process, and the fibrous actin, after standing for 30 min or longer, is again sedimented by centrifugation for 3 hr at 30,000 rpm.

The sediment is dissolved in 200 ml of 4×10^{-4}M ATP solution by stirring. This solution is dialyzed with continuous slow stirring for 2 days in the cold (some toluene is added) against 2 changes of 10^{-4}M ATP solution at pH 8.2, in an atmosphere of nitrogen or helium. The completeness of depolymerization is indicated by testing for the absence of flow birefringence; dialysis is continued if required. The solution is filtered through paper, and is centrifuged for 2 hr at 30,000 rpm.

PREPARATION OF PURIFIED MYOFIBRILS

The isolation of myofibrils in bulk is a relatively recent development. The simplest and most straightforward procedure is that of Perry (16).

Rabbit muscle is minced in the usual manner. The ground tissue is homogenized for 2 min in a Waring Blendor in 8 vol of 0.08M borate solution of pH 7.1 in the cold. The homogenate is centrifuged at 0° C for 15 min at 600*g*, and the sediment is again suspended in the same amount of borate solution as had originally been used; this is once more homogenized in the blendor for 2 min. The suspension is centrifuged in 0.1M KCl. Several more cycles of centrifugation (20–30 minutes at 600*g*) and resuspension are carried out. The suspension media are probably arbitrarily chosen and may be replaced by other media if the need arises.

PREPARATION OF TROPOMYOSIN (17)

Minced rabbit muscle is homogenized with an equal volume of cold water for 1 min in a Waring Blendor. The extract is drained off through gauze and an equal volume of ethanol is added to the residue; the fluid is again pressed out and replaced by 4 vol of 50% ethanol; the residue is then washed twice with similar volumes of ethanol and twice with ether, and is dried in the air to remove most of the ether. The fiber is then suspended in 7 vol of 1M KCl and the pH adjusted to 7 with NaOH or KOH. After extraction for 12 hr at room temperature, the fluid is filtered off through gauze and the residue is re-extracted with the same volume of 1M KCl for 2 hr. The combined filtrates form the crude tropomyosin extract. The rest of the procedure is done in the cold.

The solution is acidified to pH 4.3 with 1N HCl and the precipitate is centrifuged down after 1 hr. It is dispersed in 5 vol of water

and redissolved (except for a denatured residue) by neutralization to pH 7. Without clarification of the suspension, 0.7 vol of saturated ammonium sulfate (neutralized with 0.01 vol of concentrated ammonia) is added with stirring. The fluid is now centrifuged or filtered by suction with a filter aid. Solid ammonium sulfate is added to 0.70 saturation to precipitate tropomyosin. This is dissolved in water and dialyzed in water for 1 day. From the solution, tropomyosin is again precipitated by acidification to pH 4.5, and the collected precipitate redissolved at pH 7. The ammonium sulfate precipitation is repeated, and the fraction between 0.45 and 0.70 sat is recovered. The protein may be stored in the cold as an ammonium sulfate paste which can be dissolved in water and dialyzed; the protein is precipitated from the neutral aqueous solution with 1.5 vol of ethanol, dried with ethanol and ether, and kept as such.

Tropomyosin may be crystallized. Starting with the ammonium sulfate paste, it is mixed with an equal volume of water and dialyzed against water until complete dissolution, then dialyzed in the cold against a solution which contains 16 g of ammonium sulfate per liter in 0.01M acetate buffer of pH 5.4; small batches of external solution are used and are changed twice a day. Crystals are deposited when the pH drops below 6.0. Crystallization should be carried out only after previous purification since it does not constitute a good purification process by itself. The yield may approach 0.5 g/100 g of muscle.

PREPARATION OF A PARTICULATE ADENOSINETRIPHOSPHATASE (*18, 18a*)

Minced muscle is mixed in the cold with 5 vol of an extractant of the following composition: 0.1M KCl, 0.04M $NaHCO_3$, 0.01M Na_2CO_3 and 0.001M KCN, and is homogenized in a Waring Blendor for 1 min. After standing for 30 min, the suspension is centrifuged (15 min at 4,500 rpm in a refrigerated angle centrifuge); the sediment is re-extracted twice in the same fashion. The combined extracts are diluted with an equal volume of 0.001M KCN and recentrifuged. The particulate enzyme is then precipitated with 0.35 satd ammonium sulfate (neutralized to pH 8 with ammonia) washed once with 0.35 satd ammonium sulfate at pH 8, and then dissolved in 0.2M KCl to a volume equal to the original muscle volume. The precipitation with ammonium sulfate is repeated, the sediment now being dissolved in 0.2M KCl, 0.06M histidine (to pH 7.5) and 0.001M KCN. This solution is clarified by centrifugation at 6,000*g* for 20 min, and the particulate ATPase is then sedimented at 18,000*g* during 2–3 hr centrifugation. It is resuspended in a smaller volume of the KCl–histidine–KCN medium, forming an opalescent pink-brown solution.

PREPARATION OF ENZYMES OF THE MYOGEN FRACTION AND OF SOME OTHER ENZYMES

Among the many enzymes present in muscle besides the structural proteins already discussed, we have selected those which, in addition to their intrinsic interest, are also of technical importance as analytic reagents for the quantitative determination of muscle metabolites in very small quantities. Most of these enzymes occur in the myogen fraction and are hence obtained by aqueous extraction of minced muscle. Single enzymes have often been isolated from such extracts in a greater or lesser state of purity; recently, however, emphasis has been placed on the development of inclusive fractionation schemes in which several enzymes are obtained serially from one and the same extract. Since these fractionation procedures will no doubt be further improved during the next few years, a complete description is not considered timely, and only some selected examples will be given.

Minced muscle is extracted with 2 vol of water of 0.03N KOH, and re-extracted with 1 vol of water, as described previously. The extract serves for the isolation of water soluble enzymes, but at least one enzyme, adenylate deaminase, water soluble when isolated, is retained by the residue to a considerable extent.

Most of the water soluble enzymes can be stored for several months when kept frozen at about −20° C.

Assay procedures for the different enzymes are not described separately, since they can generally be derived from the analytic procedures described later.

PREPARATION OF ADENYLATE DEAMINASE (19)

The muscle residue remaining after extraction of the enzymes of the myogen group is washed 4 times, each time with 4 vol of cold 0.85% NaCl, for 20 min with stirring. After each washing, the fluid is drained off through 4 layers of gauze; the washings are discarded. The residue is then extracted in the cold for 1 hr with an equal volume of 2% $NaHCO_3$, whereupon the extract is obtained by filtration by suction through a Whatman No. 4 or 12 paper. The residue can still serve for the preparation of actin.

To 0.5–1 liter cold extract, 50 ml of an aged suspension of alumina Cγ are added (20–25 mg/ml) and the enzyme is allowed to become adsorbed by stirring for 15 min in the cold. The gel is then collected by centrifugation at low temperature. For elution of the enzyme from the adsorbent, prepare a solution of 1M Na_2HPO_4, warmed to about 40° C; add 50 ml of this solution to the cold centrifuge glass containing the alumina gel, so that the final tempera-

ture is about 25° C. Stir for 15 min at this temperature, then centrifuge the suspension without refrigeration. Store the extract in the cold until the bulk of the Na_2HPO_4 has crystallized; if crystallization is delayed, it can be induced by scratching the inner wall of the flask with a glass rod. Filter the solution off through a fluted paper, and wash the retained crystal mass a few times with small volumes of cold water, which are added to the first filtrate.

For each 10 ml of filtrate, add 3.7 ml of cold saturated ammonium sulfate, neutralized with 0.01 vol of concentrated ammonia (final concentration, 0.27 sat). Let stand for 10 min and remove the precipitate by high speed centrifugation, or by filtration by suction through paper and filter aid. By adding 4.5 ml of cold neutralized ammonium sulfate for each 10 ml of original solution, the ammonium sulfate concentration is raised to 0.45 sat; collect the precipitate so obtained by high speed centrifugation and dissolve in 0.1M Na_2HPO_4 (10 ml if 1 liter original extract was used).

PREPARATION OF ADENYLATE KINASE FROM AQUEOUS MUSCLE EXTRACT (*20*)

The aqueous extract is prepared as described with 0.03N KOH. It is acidified with 0.05 vol of 1N HCl, rapidly heated to 90° and kept at that temperature for 1-2 minutes, and rapidly cooled again to room temperature. The solution is then neutralized with 1 or 2N NaOH or KOH, and the resulting precipitate of denatured proteins is filtered off. Further steps are done at low temperature. Ammonium sulfate is added to 0.8 sat, the precipitate is collected by filtration on Whatman paper No. 5, and is dissolved in water to $^1/_5$ of the original extract volume.

Add $^1/_6$ of a suspension of alumina Cγ (25 mg/ml) to the solution, and keep the suspension for 1 hr at room temperature. The gel is then centrifuged off, removing contaminating protein. This step is somewhat unpredictable; apparently, the presence of a certain amount of residual ammonium sulfate is necessary to prevent adsorption of myokinase too.

The solution is cooled again, and $^1/_{40}$ vol of 10% trichloroacetic acid is added drop by drop. The precipitate is discarded; $^1/_{20}$ vol of trichloroacetic acid is added in the same manner; the precipitate is collected, and suspended in water at pH 6; the solution is dialyzed against 2% neutralized ammonium sulfate. The treatment with trichloroacetic acid may be repeated.

Adenylate kinase so prepared may still be contaminated with nucleoside diphosphokinase. This can be inactivated by heating the solution for 2 min at 100° C at pH 6 in 0.05M succinate in the presence of 10 mg glutathione per ml (21).

PREPARATION OF PYRUVATE KINASE (22, 23)

Extraction of this enzyme is not with water but with 3 vol of cold 0.1M KH_2PO_4. The extract is obtained by centrifugation or by filtration through gauze, and the enzyme is precipitated in the cold by slow addition of acetone previously chilled to $-20°$ (670 ml/-liter extract). The precipitate is collected by centrifugation after standing for 30 min. The precipitate from 1 liter extract is dissolved with 50 ml of 0.05M phosphate buffer of pH 7.2. This extract is kept for several hours in the cold in a vacuum desiccator over silica gel, to remove the acetone; it is then brought to 60 ml with water and clarified by centrifugation. After dilution with 180 ml of cold water, 112 ml of chilled acetone are added and the precipitate is collected by centrifugation and dissolved in 10 ml of water with a few drops of 0.1N KOH. This solution is diluted to 120 ml, 1.5 g of aged calcium phosphate gel are added, and the suspension is brought to 300 ml final volume and stirred for 10 min at room temperature. The gel is removed by centrifugation, and the supernatant solution is adjusted to pH 4.6 with 1M acetate buffer of pH 4, and clarified by centrifugation. After adjusting the volume to 300 ml, 333 ml of cold saturated ammonium sulfate are added, and the precipitate is removed; a further 67 ml of saturated ammonium sulfate are added and the precipitating enzyme (the solution may have to be slightly warmed at this point) is collected by centrifugation and dissolved to 20 ml with 0.02M phosphate buffer, pH 7.2.

PREPARATION OF CREATINE KINASE

Two different methods of preparing this crystalline enzyme have been described by Kuby *et al.* (24), of which only the shorter one will be given here; admittedly, this leads less readily to a crystalline preparation than the longer procedure, but once crystals have been obtained, they can be used to seed subsequent preparations.

Minced muscle is extracted (and briefly homogenized in a Waring Blendor) with 2 vol of 0.01M KCl for 15 min in the cold, and the extract is filtered off through gauze. Solid ammonium chloride is added to the extract to a concentration of 0.1M and the pH is adjusted to 9.0 with 5M NH_4OH; this causes precipitation of inorganic salts as $Mg(NH_4)PO_4$, which is completed after 30 min in the cold; 1.5 vol of cold ethanol are added, and the solution is stirred for 2 $^1/_2$ hours at 20° C; denatured proteins and inorganic salts are removed by centrifugation.

To the yellowish solution obtained in this manner, add 2.0M $MgSO_4$ (pH 8.5) to a final concentration of 0.03M, followed by cold 95% ethanol, 1.5 times the volume of the added $MgSO_4$. After

stirring at 20° for 30 min, collect the precipitate by centrifugation, and extract twice by resuspension in 0.07M magnesium acetate, pH 9.0, in volumes amounting to 6 and 4% respectively, of the original extract volume. After each extraction, the insoluble part is centrifuged off. The volumes of Mg-acetate solution used and of final solution obtained are measured accurately, in order to calculate the residual ethanol concentration in the solution.

At 0° (pH 8), cold ethanol is slowly added to a final concentration of 36% of 95% ethanol. The precipitate is removed by centrifugation after 30 min at 0°. Ethanol is added to a final concentration of 50%, and the precipitate is collected after 30 min at 0°. It is dissolved in 30–50 ml of 0.05M ammonium citrate, pH 9, any insoluble residue being centrifuged off. This solution is dialyzed against two changes of 6 liters of water with 2 ml of 5N ammonia. The dialysate is slowly brought to 56% ethanol at 0°, and the ammonia concentration is brought to 0.003N. This solution is kept at −10° C in a closed flask, which is occasionally opened and exposed to a CO_2 atmosphere (as in a dry ice storage box) until crystallization occurs. If crystallization is not desired, the preparation can be stopped before this step, at which the purity is already high.

PREPARATION OF SEVERAL SOLUBLE ENZYMES BY CORI'S INCLUSIVE FRACTIONATION PROCEDURE

Starting with an aqueous muscle extract (1 part of rabbit muscle extracted with 2 vol of 0.03N KOH, followed by re-extraction with 1 vol of same), several enzymes can be obtained successively by procedures developed by the Cori's and their collaborators. Some of these will be described.

FIRST FRACTIONATION: CRYSTALLIZATION OF ALDOLASE (25)

The pH of the muscle extract is adjusted to 7.5. Inert proteins are first removed by precipitation with ammonium sulfate, 0.5 sat, which has been saturated at room temperature (and neutralized to pH 7.5 with ammonia). It is added slowly to the extract with continuous stirring in a cold bath, so that the temperature remains 0 to 3° C. The precipitate is removed by filtration (suction filtration with filter aid, or filtration under positive pressure). To each 100 ml of filtrate, 4 ml of neutralized (pH 7.5) saturated ammonium sulfate are added, to bring the saturation to 0.52. The solution is then slowly warmed up to 18° by stirring at room temperature, to initiate crystallization, and is then placed in the cold room. Crystallization of aldolase will start within a day, but several

days are needed for a complete yield. The crystal mass is then collected by centrifugation. For the recrystallization and for assay procedures, see the original report (25). The enzyme, which catalyzes the reaction:

fructose-1,6-diphosphate $\rightleftarrows$ ph-glyceraldehyde + ph-dihydroxyacetone

has not been widely used for analytic purposes, but would appear to hold promise for such applications in combination with other enzymes (84). Its crystallization is described here because its complete separation is necessary as a preliminary to the isolation of other enzymes.

PREPARATION OF SO-CALLED 52-72 PASTE

Several enzymes can be prepared from the fraction precipitating between 0.52 and 0.72 sat with ammonium sulfate. In addition to the separation of lactic dehydrogenase from this fraction, described below, other enzymes have also been obtained.

The preparation of this group of enzymes is contingent upon the previous removal of those precipitated below 0.52 sat. Since the complete crystallization of aldolase may require up to a week, during which time some enzymes may deteriorate, the separate preparation of aldolase may be foregone and neutralized ammonium sulfate solution added, as described, to 0.52 sat at once. Aldolase then precipitates amorphously with the other proteins, and these are removed by filtration.

To the filtrate, add 130 g of solid ammonium sulfate per liter, and stir the solution in the cold (28). Collect the precipitate in the cold room by filtration by suction, without filter aid, until fluid no longer emerges, but without undue drying of the filter cake. Then invert the filter over a clean glass plate and draw the paper off so that the paste can be scraped into a container; it can be stored as such in the cold for a considerable time.

PREPARATION OF LACTIC DEHYDROGENASE

This is one of the enzymes that can be obtained from the 52–72 paste, but its preparation from this source has not been fully documented. The procedure given here has evolved in our hands from information given by several investigators (22, 26, 27).

Dissolve about 10 g of paste in 16 ml of water, and add about 7 ml of saturated ammonium sulfate in the cold until a faint turbidity appears. Then slowly warm the solution to 18 to 20° C, at which point precipitation will occur. Centrifuge the precipitate off at room temperature and redissolve in water to 3 to 5 ml. Dialyze this solution in the cold against a large volume of ammonium sul-

fate, 0.55 sat, transfer the dialysate to a small centrifuge tube and slowly bring to room temperature. Centrifuge the precipitate off and redissolve in cold water. For further purification, the solution may be heated for 3 min at 58°, the inactive precipitate removed, and ammonium sulfate added in the cold until incipient turbidity. The enzyme may then slowly crystallize, or can be made to precipitate more rapidly at room temperature. It is dissolved in 3 ml of water and stored in the frozen state.

PREPARATION OF PHOSPHOGLYCERALDEHYDE DEHYDROGENASE (28)

The muscle extract is brought to 0.72 sat, as described earlier, and the precipitating 52–72 fraction is removed by filtration. The pH of the filtrate is about 7.5. Add slowly, under continuous stirring, 15% ammonia until the pH is 8.3. Keep the solution in the cold overnight, and for several days longer, during which time a large yield of crystals appears. Collect the precipitate by filtering through a fluted filter and dissolve in water (12.5 ml for each 100 g of muscle). Clear the solution by centrifugation. Add slowly 2 vol of ammonium sulfate solution (pH 8.3, saturated at room temperature, but the enzyme solution being maintained at low temperature in a cold bath). Crystallization will be complete in a day, and is repeated 2 times in the same fashion.

For the details of the enzyme assay, see the original report (28). The assay is based upon the reduction of DPN by glyceraldehyde phosphate under the influence of the enzyme in the presence of arsenate (in order to make the reaction proceed to completion; with phosphate instead of arsenate, an equilibrium with diphosphoglycerate would be reached). This assay also indicates an important analytic application of this enzyme: determination of glyceraldehyde phosphate by the above method; of phosphodihydroxyacetone, by the addition of triosephosphate isomerase; of fructose diphosphate by the addition of aldolase; etc. Such methods, however, do not seem to have been worked out in detail.

PURIFICATION OF SEVERAL SOLUBLE ENZYMES

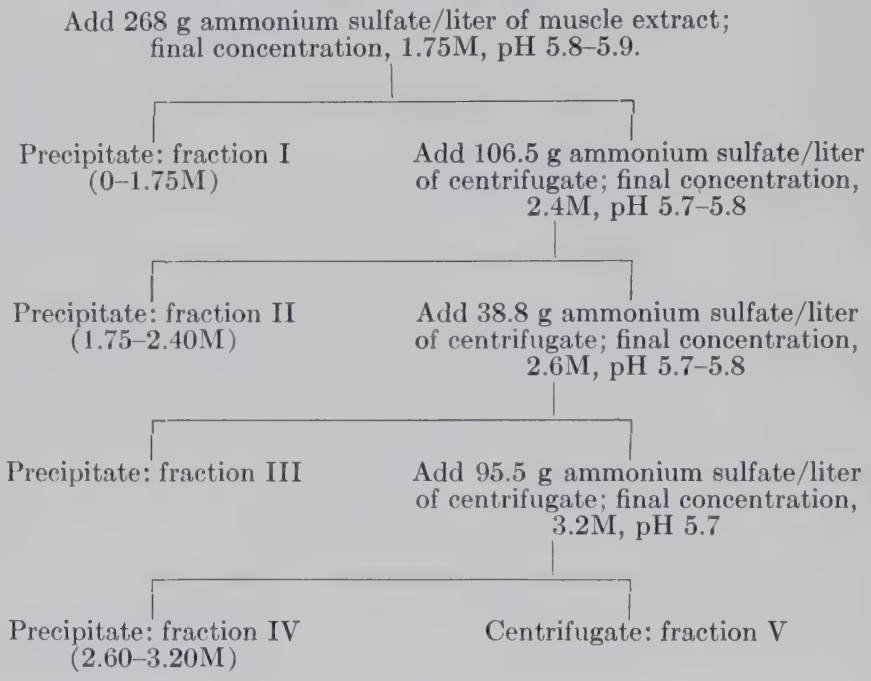
Beisenherz *et al.* (29) have described the separation of several enzymes, in the crystalline state, from an aqueous muscle extract. The methods are frequently similar to those developed in Cori's laboratory. They differ mainly in effecting the initial separation at different pH and salt concentration; in systematically excluding heavy metal impurities by the addition of Versene during the initial fractionation and the use of pure water and ammonium sulfate in later steps; and in certain technical procedures. Somewhat sharper

separations and greater yields are claimed to be obtainable than with older methods. It may be expected that the investigators in Büchner's laboratory will further develop their scheme to isolate still more enzymes. Their work will therefore not be presented in full detail here. The following parts seem useful as a general outline, and some of our experiences have been added.

SEPARATION OF FIVE INITIAL FRACTIONS

Finely minced muscle is extracted in the cold with 2 vol, and reextracted with 1 vol, of water containing 0.5 g Versene per liter, each extraction lasting 30 min. Fractionation of this extract is achieved by stepwise addition of solid ammonium sulfate in the cold. This is done slowly under constant stirring, each addition of salt lasting one to several hours (the exact time appears immaterial). For the gradual addition of salt, an automatic salt shaker can be constructed, but is not essential.

The following flowsheet describes the fractionation:


Add 268 g ammonium sulfate/liter of muscle extract; final concentration, 1.75M, pH 5.8–5.9.
Precipitate: fraction I (0–1.75M)
Add 106.5 g ammonium sulfate/liter of centrifugate; final concentration, 2.4M, pH 5.7–5.8
Precipitate: fraction II (1.75–2.40M)
Add 38.8 g ammonium sulfate/liter of centrifugate; final concentration, 2.6M, pH 5.7–5.8
Precipitate: fraction III
Add 95.5 g ammonium sulfate/liter of centrifugate; final concentration, 3.2M, pH 5.7
Precipitate: fraction IV (2.60–3.20M)
Centrifugate: fraction V

Products from Fraction II. Preparation of Lactic Dehydrogenase

Fraction I has so far not been further resolved (presumably this fraction contains phosphorylase and other enzymes). Fraction II has been reported to yield aldolase, α-glycerophosphate dehydrogenase, and lactic dehydrogenase, by stepwise increase of the ammonium sulfate concentration. Because of its analytic applications,

the preparation of lactic dehydrogenase will be described, including some modifications introduced in this laboratory.

70 ml of precipitate of fraction II was brought to 170 ml final volume with 100 ml water, to which 50 ml Versene and 1 ml of 0.5M acetate buffer, pH 4.6, were added. The pH was kept adjusted to 6.2, and 15.5 g ammonium sulfate were added. After standing overnight and centrifugation (164 ml of supernatant solution), a further 8.6 g ammonium sulfate were added to a final concentration of 1.75M, and the pH maintained at 6.2. After again standing overnight and centrifugation, the solution was dialyzed against 2 changes of 1.82M ammonium sulfate, pH 6.2; in the same fashion, through a step of 2.1M, the solution was finally dialyzed to equilibrium with 2.30M ammonium sulfate, pH 6.2. The precipitate which formed after 4 days was removed by centrifugation.

The enzyme solution was now dialyzed for 2 days against 2.32M ammonium sulfate, pH 6.2; a precipitate formed, which was greatly enhanced by slowly warming the system to about 10° C. It was suspended in 20 ml of 1.8M ammonium sulfate. To this were added 0.9 ml of 0.2M Na-pyrophosphate, 0.9 ml of 1N ammonia, and 0.6 ml water, and the system was gently stirred in the cold to dissolve the protein; 1.9 g ammonium sulfate were added, the precipitate was collected by high speed centrifugation, washed 3 times with 10 ml of 2.0M ammonium sulfate and 0.02M ammonia, and dissolved in 10 ml of water. This preparation was free of demonstrable amounts of pyruvate phosphokinase, which, before the last reprecipitation and washings, was present in significant amounts.

Products from Fraction III. Pyruvate Phosphokinase

This preparation will also be described in the form of a protocol.

28 ml of centrifuged precipitate of fraction III were dissolved in 50 ml of 0.02M acetate buffer, pH 4.6, with Versene, to 78 ml volume; over a period of 2 hr, 10.5 g ammonium sulfate and 1 ml of 0.5M acetate buffer, pH 4.6, were added with stirring in the cold. The container was left overnight in the cold, during which pyruvate phosphokinase crystallized; the yield was increased by dialysis for 2 days against 1.95M ammonium sulfate. The precipitate was collected by centrifugation, washed with 1.95M ammonium sulfate, and dissolved in 35 ml water; 10 g ammonium sulfate were added, and the solution was immediately clarified by centrifugation. Pyruvate phosphokinase precipitated from the supernatant after several days standing in the cold. The enzyme was dissolved in 10 ml water.

FRACTIONATION OF SOLUBLE MUSCLE ENZYMES WITH ORGANIC SOLVENTS

The separation of muscle enzymes with organic solvents has not been worked out in full detail, but the experiences reported by Askonas (30) indicate that further work in this direction is to be recommended.

The starting material is an extract obtained by incubating ground muscle at 0° for 20 min with an equal volume of water. The extract obtained after centrifugation is then dialyzed overnight against a large volume of distilled water, so that its conductivity is reduced to about that of 0.007M KCl solution. After centrifugation and adjustment to a certain pH, fractionation is then effected by slowly adding methanol, ethanol, propanol or acetone through a capillary, the solution being stirred constantly in a cold bath. Initially, a bath at −3° C is used; later, at higher solvent concentration, the temperature is lowered to −5 or −7° C. The precipitates are rapidly collected by centrifugation, dissolved and dialyzed.

In general, acetone seems to be the best solvent for initial separations, but propanol has been found especially useful for obtaining enzymes that resist destruction by this alcohol. Several workers (31, 32) have used creatine phosphokinase prepared in this manner. This is made by adjusting the pH of the dialyzed extract to 6.5, and adding *n*-propanol to a concentration of 50% by volume; the precipitate so formed is removed by centrifugation; *n*-propanol is further added to a final concentration of 60%, and the precipitate formed in this step is dissolved in water, dialyzed and stored in the cold. This enzyme is not pure, but is obtained in good yield with little effort, and is most useful in studies in which complete purity is not essential.

ANALYSIS OF MUSCLE PROTEIN FRACTIONS

No special procedures will be outlined for the water soluble protein fractions. If desired, the total amount of water soluble protein can be determined by extraction of a weighed amount of minced muscle. However, the determination of myosin and actin are described briefly, on account of the special problems of extraction and separation.

QUANTITATIVE EXTRACTION OF MYOSIN (85)

This method has been developed for rabbit muscle, and may conceivably need some modification before being applied to other materials. It is based upon the complete extraction of myosin by a salt solution containing pyrophosphate (which, like ATP, dissociates myosin from the fibrous structure), followed by extraction of actin from the finely divided material at alkaline reaction.

Muscle is cooled and minced, first through a meat grinder, then with a Latapie mill or its micro-modification.* The mince is extracted in the cold for 20 min with 10 vol of 0.47M KCl, 0.01M K_4-

* Arthur H. Thomas Co., Philadelphia.

P_2O_7 and 0.01M phosphate, final pH 6.2. After centrifugation, the residue is re-extracted 4 times, each time with 4 vol of the same extractant, for 1–2 hr.

The extracts are combined. An aliquot is diluted 15-fold with water; after standing 1 hr or longer, the precipitated myosin is centrifuged off, and its amount determined with one of the methods described below. For accurate work, it may also be necessary to determine the protein or nitrogen in the supernatant solution, and to correct for the amount of this which is enclosed in the myosin pellet; or the myosin precipitate can be washed with a small amount of 0.04M KCl.

QUANTITATIVE EXTRACTION OF ACTIN

After the complete extraction of myosin, the residue is suspended in 6 vol of 0.6M KCl, and the pH adjusted to 7.0. The suspension is homogenized in a Waring Blendor for 2–4 min, and is left in the cold for 18 hr. After centrifugation and brief re-extraction, the protein content of the extracts, which is actin, is determined.

Direct extraction for 18 hr of minced muscle with pyrophosphate-KCl at pH 7, after homogenization in a Blendor, would presumably yield a measure for the total actomyosin content, but no systematic studies on this subject seem to be available.

DETERMINATION OF PROTEIN CONTENT

The amount of protein present in the myosin and actin fractions can, of course, be determined with any of the available nitrogen determination methods. (We prefer the Kjeldahl procedure according to Hiller *et al.* (86).) The nitrogen content of myosin and actin, respectively, are 16.2 and 15.2% (2, 15). For many less exacting purposes, however, the biuret method can be strongly recommended; we employ the Weichselbaum-Kirk method (70) as modified by Beisenherz *et al.* (29). It will be described here for the determination of total myosin plus actomyosin in a muscle extract obtained by extraction with Guba-Straub solution. Aliquots of 0.5 ml extract are added to 5 ml cold water, and precipitation is allowed to occur for 2 hr or more. After centrifugation, the supernatant solutions are decanted, and the inverted tubes are left to drain on filter paper. Each pellet is suspended in 3 ml water, with a thin glass rod left in the tube, and 3 ml of biuret reagent are added (dissolve 9 g K Na tartrate in 400 ml of 0.2N NaOH; dissolve herein subsequently 3 g powdered $CuSO_4 \cdot 5H_2O$ and 5 g of KI; make up to 1 liter with 0.2N NaOH; store in a brown bottle). After 30 min at room temperature, the color is read at 540 mμ; standardization is done against an actomyosin solution of known concentration treated similarly.

II. QUANTITATIVE DETERMINATION OF SOME SUBSTANCES INVOLVED IN ACTIVITY METABOLISM

EXTRACTION OF MUSCULAR TISSUE FOR DETERMINATION OF ACID SOLUBLE COMPOUNDS

The initial step in every extraction for analytic purposes is sufficient disintegration of the tissue. Unfortunately, this may be a difficult procedure in the case of muscle, especially when performed on a small scale (e.g., in biopsy samples, or on small muscles employed in physiologic experiments, such as the papillary muscle preparation or small skeletal muscles of frog or turtle). On the one hand, most muscular tissues are tough and fibrous, and do not lend themselves well to grinding, although there are some notable exceptions; on the other hand, the disintegration has to be performed in such a manner as to exclude secondary chemical changes. A procedure suitable for all cases cannot be given. Often, however, freezing the tissue rapidly, crushing it in the frozen condition, and extracting it immediately without permitting it to thaw will be found advisable.

While it will not suit all cases, brief reference will be made to the technic employed in the analysis of small (150–300 mg) skeletal muscles of turtles (33, 34). These are instantaneously frozen by rapid immersion in liquid propane or liquid propane mixed with $^1/_4$ part of isopentane, or with pure isopentane (grade "pure" from Phillips Petroleum Company, Bartlesville, Oklahoma) which has been cooled to about $-185°$ C with liquid nitrogen (liquid air should not be used to cool hydrocarbons because of grave explosion danger). Wrapped in parafilm to prevent condensation of water vapor from the air, and to provide thermal insulation, the muscles are weighed on a torsion balance, crushed at liquid nitrogen temperature in a closed steel crusher, and quantitatively transferred to a specially designed tube: in this tube, the coarsely broken-up tissue is first lyophilized, ground with a glass pestle, and then extracted in the cold with perchloric acid (see below). While such extensive precautions are not always necessary, careful consideration of the problems involved in extraction is always required.

Most analyses of metabolic intermediates are performed on deproteinized extract. As a protein precipitant, trichloroacetic acid

(TCA) has long been favored and remains useful (TCA can be removed by extraction with ether. More recently, perchloric acid (PCA) has come into use (35). Its advantages are: *1*) PCA solutions are perfectly transparent in the ultraviolet, whereas TCA has absorbing impurities; *2*) since $KClO_4$ is only sparingly soluble in cold water, its neutralization with KOH provides a means for obtaining an extract low in salt, which is especially important for chromatographic analyses; *3*) if neutralization with KOH under careful control of the pH is too time consuming, or is impractical with small volumes of solution, this neutralization can be effected with a slight excess of the calculated amount of $KHCO_3$, while $NaHCO_3$ is used in the same fashion when removal of the perchlorate is not imperative. We usually extract 150 to 400 mg of muscle with 2 + 1 ml of 0.25N PCA, and after centrifugation in the cold, collect the supernatant solutions in a vessel containing 1 ml of 0.8M $NaHCO_3$. Dissolved CO_2 is then removed from the neutralized solution by a brief evacuation under a bell jar.

For the extraction of substances that are very labile toward acid, we now employ 75% methanol as the deproteinizing extractant. This is applied to crushed frozen muscle at −60° C, and is subsequently removed by vacuum evaporation.

SOME CHEMICAL DETERMINATIONS

INORGANIC PHOSPHATE DETERMINATION (36)

Reagents.—*1*) Acid molybdate (so-called molybdate I): Dissolve 25 g ammonium molybdate in some water in a 1 liter volumetric flask; add 500 ml of 10N H_2SO_4 (450 ml of concentrated H_2SO_4 slowly added to 1,300 ml water and the normality checked by titration) and fill up to 1 liter with water. *2*) Reducing solution: Dissolve 0.5 g of 1,2,4-aminonaphtholsulfonic acid (Eastman) in 195 ml of 15% sodium bisulfite solution (prepared a few days beforehand, and filtered if turbid), and add 5 ml of 20% sodium sulfite; shake until the powder has dissolved completely, which may require addition of more sulfite; the solution is stable in a brown bottle in the cold for about a month. Some other reducers can be used instead of the aminonaphtholsulfonic acid. *3*) Standard phosphate solution: For the stock solution, dissolve 272 mg of pure dry KH_2PO_4 in water, add 5 ml of 10N H_2SO_4, and dilute with water to 1 liter; this is stable indefinitely. Prepare a working solution by accurate 10-fold dilution with water; 5 ml of this contains 1 μM of phosphate (31 μgP).

Procedure.—In a 10 ml volumetric flask, mix an amount of extract containing a suitable quantity of phosphate, and some water;

add 1 ml molybdate solution and 0.5 ml reducer, and dilute with water to 10 ml; mix by inversion. In the same manner, prepare a reference by using 5 ml standard solution (1 μM of P). After 5 min read the absorption in the far red, e.g., at 660 or 700 mμ.

When a certain type of analysis is to be performed recurrently, it is often convenient to use the deproteinized tissue extract directly without neutralization; the molybdate solution should then correspondingly be less acid; e.g., if the sample consists of 5 ml of a filtrate of 8% TCA (obtained by extracting material with 4 vol of 10% TCA) the so-called molybdate II (made by using 300 instead of 500 ml of 10N H_2SO_4) is employed.

It would be out of place to discuss all aspects of the determination of phosphate, but one practical point should be mentioned. Like phosphate, silicate also causes a blue color. Many a mysterious result has been caused by the use, at some point in the procedure, of alkali kept in a glass bottle.

Behavior of labile phosphates in Fiske-SubbaRow method

Certain phosphate compounds are so labile that marked breakdown would occur at the acidity (0.5N) prevalent during the determination. The simultaneous presence of molybdate retards this hydrolysis in some cases (phosphoarginine and phosphoglycocyamine) while accelerating it in others (phosphocreatine and acetylphosphate). These molybdate-labile phosphates will therefore increase the apparent inorganic phosphate reading. However, their presence can be recognized because this complex hydrolysis requires more time than the color development with phosphate itself. In favorable cases, one can determine inorganic phosphate and creatine phosphate side by side by taking readings at several intervals.

Other phosphate compounds are hydrolyzed under slightly more drastic conditions, e.g., heating in acid. It is well known that 7–15 min hydrolysis in 1N HCl at 100° liberates the terminal two phosphates of ATP, and also the terminal phosphate of ADP; this treatment also splits pyrophosphate, and dephosphorylates glucose-1-phosphate. Determination of hydrolyzable phosphate once played an important role as a measure of ATP, but this method has lost much of its significance now that more specific and informative procedures are available.

Upon long standing in the Fiske-SubbaRow reagent, ATP and other substances may eventually develop some color, presumably due to hydrolysis. Pyrophosphate slowly causes a special color; the acceleration of its development by cystine has been used as a reaction for pyrophosphate (37).

SEPARATE DETERMINATION OF PHOSPHATE AND PHOSPHOCREATINE (38)

True inorganic phosphate and phosphocreatine can be determined side by side after precipitation of the former as the calcium salt. This is achieved by adding 0.2–0.25 vol of a 10% $CaCl_2$ solution, saturated with $Ca(OH)_2$ at pH 8.8 to the neutralized extract. After 10–15 min standing at room temperature, the phosphate precipitate is removed by centrifugation, washed with some of the 5-fold diluted $CaCl_2$–$Ca(OH)_2$ solution, and dissolved in the minimal amount of HCl. Phosphate is then determined directly in this extract. Phosphocreatine is obtained as the difference between the "true" and the "apparent" inorganic phosphate, or can be determined separately in the solutions after calcium precipitation after passing this solution through an ion exchange column in the potassium or sodium cycle to remove the excess calcium.

PHOSPHATE DETERMINATION IN PRESENCE OF MOLYBDATE-LABILE PHOSPHATE COMPOUNDS (39)

This method proceeds on the same general lines as the Fiske-SubbaRow procedure, but works at low acidity. Therefore, the acid molybdate-labile compounds such as phosphocreatine interfere much less with the determination, and although the color continues to increase on their account it does so more slowly and the effect can be excluded more satisfactorily.

Tissue extracts obtained with trichloroacetic or perchloric acids are rapidly neutralized to pH 4 to 4.2 by adding, for example, 4 vol of 0.1M sodium acetate solution. In case of very labile phosphates, one may deproteinize with saturated ammonium sulfate, buffered to pH 4 with 0.1N acetic acid and 0.025M sodium acetate. An aliquot of the filtrate is placed in a flask and diluted with acetate buffer (0.1N acetic acid, 0.025M sodium acetate) to a suitable range of phosphate values (ammonium sulfate extracts should be diluted at least 5-fold). For each ml of final solution, add 0.1 ml of 1% ascorbic acid solution (fresh) and 0.1 ml of 1% ammonium molybdate in 0.05N H_2SO_4. A blank and a standard are treated in the same fashion. The color is read after 5 min and the phosphate content calculated by comparison with the standard. A second comparison is made after 10 min, and the increase of apparent phosphate, if any, is subtracted from the 5 min value in order to obtain the true inorganic phosphate value.

Lowry and Lopez (39) have applied the method successfully to muscle, but we have found it entirely unpredictable for crude actin extracts. The procedure seems to be affected by sulfhydryl com-

pounds, and its applicability to new uses must therefore be carefully tested.

INORGANIC PHOSPHATE AND PHOSPHOCREATINE DETERMINATION BY BUTANOL EXTRACTION METHOD

An important modification was introduced in the methodology of phosphate analysis by Berenblum and Chain (40) who added acid and molybdate to the sample and removed the phosphomolybdate so formed by extraction with isobutanol. Shaking the isobutanol layer with a reducer, stannous chloride, then led to the development of the blue color. This method is more sensitive than the Fiske-SubbaRow method, and, since the phosphomolybdate is freed of other water soluble constituents, the procedure is less subject to the manifold disturbances affecting the stannous chloride method when applied directly. The description which follows is Ennor and Stocken's (41) modification of the original method. One extracts, first, phosphomolybdate rapidly at the minimal sufficient acid and molybdate concentrations, to prevent breakdown of phosphocreatine; after more prolonged treatment with stronger acid and molybdate, the sum of inorganic phosphate and of phosphocreatine is obtained. Alternatively, the aqueous phase of the first determination could be used to perform the second one, but this has not been worked out systematically, and may be somewhat less easy to perform with precision.

Reagents.—1) Ammonium molybdate: dilute, 1%; concentrated, 5%. *2)* Sulfuric acid: dilute, 1N; concentrated, 10N. *3)* NaCl, 5%. *4)* Isobutanol. *5)* Stannous chloride stock solution: 10 g of $SnCl_2$ in 25 ml of concentrated HCl, kept in the dark in the refrigerator; immediately before use, dilute 0.5 ml with 100 ml of 1N H_2SO_4.

Procedure for Inorganic Phosphate

Place the neutralized sample to be analyzed in a separatory funnel, and add water to a total volume of 6 ml; also set up another funnel (blank) with 6 ml of water, and one with standard phosphate, e.g., 0.4 μM of phosphate, likewise made up to 6 ml. The following operations are to be performed in rapid sequence, and it is recommended that all reagents be added by squirting them from automatic pipets, such as the commercially available syringe type of pipet.* Add to a funnel 2 ml of 5% NaCl (this serves to speed up the separation of the phases after shaking), 1 ml of 1N H_2SO_4 (the 2 fluids can also be mixed beforehand and added at once), and 1 ml

* Bicknell Associates, Cambridge, Mass.

of 1% ammonium molybdate. After 20 sec, squirt 20 ml of isobutanol into the funnel, and shake the contents for 10 sec, after which the phases are allowed to separate; the aqueous (lower) phase is drained off, and the butanol layer is washed twice by injection of 10 ml of 1N H_2SO_4 followed by shaking or swirling for 10 sec and removal of the aqueous layer. The alcohol layer is then shaken for 1 min with 20 ml of dilute $SnCl_2$, to reduce the phosphomolybdate under formation of the blue color. After phase separation, the acid layer is again discarded, the butanol layer is collected in a 25 ml volumetric flask and is brought to volume with ethanol. The color intensity of the solutions so obtained is compared (at 650 to 700 mμ) with that of the standard phosphate.

The volumes of solutions used can be changed somewhat to fit special needs, and the procedure works very well at a scale 0.4 times smaller than described.

Procedure for Total Molybdate-Labile Phosphate

As in the Fiske-SubbaRow method, this determination gives the "apparent inorganic phosphate," which in the case of muscle is considered as the inorganic phosphate plus phosphocreatine. The determination is carried out exactly like the one just described for inorganic phosphate, except that the concentrated solutions of molybdate and of H_2SO_4 are used, and that the final mixture is allowed to stand 15 min before adding isobutanol.

DETERMINATION OF CREATINE WITH THE DIACETYL REACTION (*42, 43*)

This method is based on the color reaction of creatine with diacetyl and α-naphthol. This reaction is also given by some related substances, such as arginine and glycocyamine, but with these the reaction is less sensitive, and their presence in significant amounts can often be excluded. We describe the procedure in its simplest form, assuming that the material to be analyzed does not contain significant amounts of sulfhydryl compounds, which would depress the color intensity. The assumption appears to be correct for most samples of skeletal muscle. A measurable degree of interference may occur with heart muscle; this may be corrected by adding a small amount of a mercurial (e.g., *p*-chloromercuribenzoate or any of the mercurial diuretics, such as Mersalyl) to the reaction mixture.

Reagents.—*1)* A stock solution of 1 ml diacetyl in 100 ml water, which can be kept in the cold for several months; a 1:25 dilution with water is prepared just before use. *2)* A freshly prepared solution of 0.5 g pure α-naphthol in 50 ml alkali (30 g of NaOH and

80 g of Na_2CO_3 in 500 ml water). *3*) Creatine standard solution, a stock solution which can be kept for several weeks at least; it contains 131 mg creatine per 100 ml water; diluted 1:100 freshly before use, the standard contains 0.1 μM per ml (13.1 μg).

Procedure.–Place in a 10 ml volumetric flask, an aliquot of the solution, containing preferably 0.1–0.3 μM of creatine. Add water to about 5 ml, then 2 ml alkaline α-naphthol and 1 ml diluted diacetyl; add water to the mark and mix by inversion. Prepare a blank containing water only, and a standard solution with 2 ml diluted standard (0.2 μM). After 15 min, the absorption is read at 520 mμ.

DETERMINATION OF PHOSPHOCREATINE WITH DIACETYL METHOD (42)

The method proceeds along the same lines as the creatine determination, with the additional operation of first hydrolyzing the phosphocreatine to be determined. This has to be performed with special precautions, since acid hydrolysis can easily lead to creatinine instead of creatine. As found by Ennor and Rosenberg (42), creatine is formed quantitatively if the hydrolysis is performed with 0.1N HCl at 65° C for 9 min. One neutralizes with a slight excess over the calculated amount of $NaHCO_3$, and proceeds as for free creatine.

ANALYSIS OF NUCLEOTIDES

No methods will be given here for the isolation and purification, or for the synthesis, of nucleotides and of other phosphorylated compounds. Increasingly, these substances are being made available commercially; even rare nucleotides, such as the polyphosphates of uridylic and guanylic acids, begin to appear on the market in good quality. Standard preparations of ATP, ADP, and AMP have been supplied for some time by a number of firms*. In the case of substances of untried origin, however, caution is required. I once procured a sample of phosphocreatine which seemed to contain no demonstrable quantity of this material nor of phosphate or creatine. A word of warning is therefore necessary to prevent a flood of reports on the effects of commercially obtained materials upon some biologic phenomenon, without prior tests of those materials. Occasionally, it may save time to derive one compound from another already on hand, by steps such as dephosphorylation or deamination. A few such examples will be given below. Otherwise we shall not enter into a treatment of this prepara-

* Sigma Chemical Company, St. Louis, Mo.; Pabst Laboratories, Milwaukee, Wis.

tive-chemical field, but refer to other collections of methods (e.g., 44, 45).

Many of the phosphorylated compounds are labile; when prolonged storage is necessary, this should be done at low temperature and in perfectly dry condition. We effect this by keeping each vial, loosely stoppered, separately in a jar tightly closed with a screw cover, with a liberal amount of indicating silica gel as a drying agent, which is kept at −20° C in a deepfreeze chest. Before use, the vial is rapidly transferred to a similar desiccator at room temperature, in which it is kept until warmed up, to preclude the possibility of a water vapor condensation. Neutral solutions of most substances can be stored for reasonable lengths of time when kept frozen at −20° C.

CONVERSION OF BARIUM OR CALCIUM SALTS OF PHOSPHORYLATED COMPOUNDS INTO SOLUBLE SALTS

At the end of an isolation procedure, phosphorylated compounds are often obtained as Ba or Ca salts. Conversion of these into soluble K or Na salts is therefore a frequent operation. This starts by suspending the dry material in some cold water, and dissolving it by the addition of a slight excess of HCl. From this solution, Ba can then be precipitated as $BaSO_4$, after which the filtrate is neutralized. However, this procedure does not always guarantee complete removal of the heavy metal, particularly since some phosphate compounds may have a solubilizing action. In particular, traces of Hg, often used in previous precipitation steps, may be carried over into the final solution, and may then interfere in the experiment by acting as an enzyme poison. For this reason, it is preferable to remove the heavy metal by an ion exchange resin, as was first practiced by Polis and Meyerhof (46). This will be illustrated by a few examples.

Preparation of Cation Resin

The resin (e.g., Amberlite IR-100 or -120, or Dowex-50, about 200 mesh) is cleaned by alternate washings with acid and base, and is brought into the desired condition by several washings with 0.5M Na_2CO_3 or K_2CO_3, followed by repeated rinsing with water. The material is then air dried and stored as such.

Conversion of Barium ATP into the K Salt

Suspend 100 mg of barium ATP in a few ml of cold water, add 5–6 ml cold 0.1N HCl, and stir until complete dissolution. A small column of potassium resin is prepared by pouring a slurry of the resin into a fritted glass filter of about 1 cm diameter until it forms

a 1–2 cm. high layer. This is washed with a small amount of water and left to drain. The ATP solution is now poured onto it, and is allowed to filter through the column by gravity; this is followed by 2 rinsings with water. The effluent usually has a pH near 4; it is further neutralized with 0.1N KOH to a predetermined pH and is diluted to the desired volume.

Conversion of the Ca Salt of Phosphocreatine into the K Salt

Phosphocreatine is usually obtained as the Ca salt, which is quite soluble in water. The aqueous solution is directly applied to a cation exchange resin column, as described. Phosphocreatine solutions are very labile in acid, but are quite stable at alkaline reaction.

Conversion of the AgBa Salt of Phosphopyruvate into the K salt

Phosphopyruvate, when synthesized, is usually obtained as the mixed AgBa salt.* Treat 200 mg with 30 ml of 0.1N HCl and stir for 5 min; decant the solution from the AgCl precipitate, which is washed with a few ml of diluted HCl which are added to the main fraction. Filter the solution through a 1 cm layer of K resin in a sintered-glass funnel of 3 cm diameter, and wash the resin with 3 portions of 5 ml water. The solution can be neutralized and used. However, if it is suspected that free pyruvate may have formed in the sample during storage, the phosphopyruvate is first reprecipitated as Ba salt. For this purpose, add 5 drops of phenolphthalein to the solution obtained as described (about 50 ml), which is neutralized with 1N KOH (about 18 drops) until faint pink. Add 3 ml of 2M barium acetate, keep the mixture alkaline with a small drop of KOH, filter, and add 2 vol of cold ethanol. Let stand in ice for 10 min, collect the precipitate by centrifugation, and dry it, by centrifugation, with several washings of ethanol and ether. This Ba salt can then be converted to the K salt by dissolution in HCl filtration through K resin, and neutralization.

DEAMINATION OF ADENYLIC TO INOSINIC NUCLEOTIDES (*47*)

As applied to ATP, this conversion is carried out as follows: Dissolve 1 g of ATP in 20 ml water; to this add 3 g sodium acetate·$3H_2O$, 6 ml of glacial acetic acid, and 15 ml of 50% $NaNO_2$; the resulting pH should be about 4, which can be controlled by adding the last portion of acetic acid stepwise while measuring the pH. After 6 hr at room temperature, the reaction mixture is cooled,

* This can be purchased from the Biochemical Research Foundation, Los Angeles, Cal.

and neutralized with 2N NaOH to pH 6.5; add 12 ml of 3M $BaCl_2$ followed by 2 vol of cold ethanol. Dissolve the precipitate of barium inosine triphosphate in 50 ml of 0.1N HCl and re-extract the residue with 20 ml of 0.1N HCl; an excess of nitrous acid may be removed at this stage by 1 hr incubation at 0° with 1 g of urea. Reprecipitate the barium inosine triphosphate by adding 25 ml of 2M barium acetate followed by 100 ml ethanol, and dry with ethanol and ether.

PREPARATION OF AMP FROM ATP

Removal of the two terminal phosphate groups is effected by alkaline hydrolysis. Of course, acid hydrolysis also effects liberation of these phosphates, but it splits the ribose moiety from the adenine as well. Contrary to a widespread belief, therefore, AMP cannot be prepared from ATP by acid hydrolysis. For a detailed account of Kerr's procedure consult the original report, or Hawk *et al.* (49), p. 283.

CHEMICAL ANALYSIS OF NUCLEOTIDES (49)

Taking ATP as an example, we shall briefly outline the main analyses performed to characterize or test a preparation. A solution is made up so as to contain about 1 mg of ATP per ml. In separate aliquots the following analyses are performed. *1) Inorganic phosphate* in 1 ml of solution, with the Fiske SubbaRow method; this should give a negligible reading. *2) Easily hydrolyzable phosphate:* Dilute 0.5 ml of the solution to 4 ml and add 1 ml of 5N HCl; place this in boiling water for 10 min (preferences of individual workers vary from 7–15 min); cool, add 1 ml of 2.5% ammonium molybdate and 0.5 ml of reducer, and proceed as for the determination of phosphate; subtract the inorganic phosphate, if any, to calculate the labile phosphate. *3) Total phosphorus:* In a large test tube, mix 1 ml of ATP solution and 1.2 ml of 10N H_2SO_4; heat this until the water has boiled off, and digest the sample until the fluid is brown; clear with 1 drop of 30% H_2O_2 followed by further boiling; transfer to a 25 ml volumetric flask and determine the phosphate with correspondingly increased amounts of molybdate and reducer. Subject a sample of the P standard to the same operations as the nucleotide solution, as well as the water blank. *4) Ribose:* Dilute 0.5 ml to 5 ml in a test tube and add 5 ml of 1% orcinol, freshly dissolved in 0.1% $FeCl_3$ solution in concentrated HCl (orcinol may need recrystallization from benzene); place this in boiling water for 30 min; cool, and read the color at 660 mμ. A standard is prepared from ribose or arabinose, diluted to contain 0.5 μM/5 ml of sample. *5) Nitrogen*, with a suitable micro-

Kjeldahl digestion and Nesslerization. *6) Spectrophotometric characterization* (see below). In the case of ATP, the ratio of adenine (spectrophotometrically) : ribose : nitrogen : total P : hydrolyzable P equals 1:1:5:3:2.

ULTRAVIOLET ABSORPTION SPECTRA

Determination of the ultraviolet absorption spectrum is an important aid in the characterization of nucleotides. Unfortunately, however, many of the standard figures still date from a time when entirely pure and reliable preparations of the nucleotides were not available, and final measurements are still needed. However, the following tabulation (Table 1) may be useful for the moment.

TABLE 1.—Approximate Spectroscopic Characteristics of Nucleotides in Acid or Neutral Solutions[a]

Solution	λ_{max}	ϵ_{max}	D 250:260	D 280:260
Adenylic	260	15[3]	0.8	0.2
Inosinic	250	13	1.6	...
Guanylic	258	12	0.9	0.7
Cytidylic	280	13	0.43	2.1
Uridylic	260	10	0.74	0.38

[a] λ_{max}, position of the absorption maximum, in mμ; ϵ_{max}, optical density of a 1 cm layer of solution of 10^{-3}M concentration; D, ratios of the optical densities at the indicated wave lengths.

ADSORPTION ON ACTIVE CHARCOAL (50, 51)

A most useful procedure is the adsorption of nucleotides on activated carbon. By this device, it is possible to concentrate nucleotides from large volumes, and to effect preliminary separation from other compounds such as phosphorylated sugars, and inorganic phosphate and pyrophosphate. The adsorption is most efficient in acid solution but can also be carried out near neutrality. Floating of the charcoal is diminished by layering some ethanol on top of the solution. Some samples of charcoal, however, even after extensive purification by boiling with acid, may cause hydrolysis of di- or triphosphates; a particular batch should therefore be tested first, by analyzing a triphosphate of known purity after its absorption and elution, e.g., by paper chromatography. Elution is achieved with 50% ethanol or with aqueous pyridine.

ION EXCHANGE CHROMATOGRAPHY OF NUCLEOTIDES

CHROMATOGRAPHY OF ADENOSINE POLYPHOSPHATES ON ANION EXCHANGE COLUMNS

Important developments in the chemistry of nucleotides have been made possible by the chromatographic procedures initiated

by Cohn and Carter (52). Their application to the analysis of nucleotides obtained by the hydrolysis of nucleic acids is beyond the scope of this review. We shall only describe the separation of AMP, ADP and ATP, worked out by Cohn and Carter for mixtures of the pure compounds, and thereupon applied by us to muscle and heart extracts (53–55).

Preparation of Tissue Extract

The ground or crushed tissue is extracted with perchloric acid and neutralized with KOH or $KHCO_3$ as described. After removing $KClO_4$ by crystallization in the cold, the extract is brought to volume (e.g., 50 ml if 10 ml initial extract was available) with water with 0.02 vol of 5N ammonia. Alternatively, the nucleotides can be absorbed on charcoal and eluted with 50% ethanol.

Preparation of Column

Although other resins may serve (or are even preferable), all work so far has been done with Dowex-1 or -2. For better retention and resolution, it is advisable to use a special low cross-linkage resin, e.g., 2 or 4% cross-linkage instead of the standard 10%. The resin is cleaned by repeated washings with acid and base, and brought into the chloride state by washing with 1N HCl, 1M KCl, and finally with water. If only the adenosine phosphates are to be analyzed, a column of 1 cm diameter and 1–2 cm high suffices.

Application and Elution of Nucleotides

The nucleotides are absorbed from the low-salt alkaline solution which is slowly filtered through the column. After a washing with water, elution is achieved with 3 serially applied solvents: 0.003N HCl, 0.01N HCl with 0.02M KCl, and 0.01N HCl with 0.2M KCl, each applied in batches of 100 ml. These elute AMP, ADP, and ATP, respectively, usually within the first 30 to 40 ml of eluent. Concentrations of each of the nucleotides are determined in the eluted fractions by determining the ultraviolet absorption at 260 mμ, and each fraction can be further characterized by determining the 250:260 and 280:260 absorption ratios and by chemical analyses of the type described in an earlier section.

Resolution of Additional Components

More detailed chromatograms can be obtained by increasing somewhat the height of the columns (e.g., 5 cm) and by using larger volumes of the eluents (200 to 400 ml). In this manner, inosine monophosphate is found to be eluted after AMP, with good

separation (54). The inosinic polyphosphates all appear well after the adenosinic derivatives, but inosine diphosphate and adenosine triphosphate overlap considerably (56) and are separated on Dowex-2 by inserting an elution with 0.01N HCl and 0.05M KCl (57). Guanosine triphosphate and uridine triphosphate have been demonstrated similarly (58). The method has been used for the study of adenylate kinase (59, 60) and of nucleotide transformations linked with the polymerization of actin (61).

Gradient Elution Chromatography

By applying solvents of increasing eluting strength, not batchwise but in such a manner that the composition of the solvent changes gradually, the sharpness of the peaks can be increased and trailing (which occurs especially with the ADP fraction) suppressed, so that better resolution of a multicompound mixture may be expected. A detailed separation of tissue nucleotides by means of formate–formic acid mixtures has been described in a recent investigation by Hurlbert *et al.* (62). Our efforts to obtain such separation with HCl–KCl mixtures have not as yet been equally successful. Further development of these methods may be expected.

SEPARATION OF ADENOSINE POLYPHOSPHATES BY PAPER CHROMATOGRAPHY

Several methods for the paper chromatography of AMP, ADP and ATP, as well as for paper ionophoresis, have been reported. If no additional compounds are to be resolved, the best one seems to be the second half of the procedure of Eggleston and Hems (63), which will be described (see Webster [64]).

A muscle extract is prepared with TCA (which can remain unneutralized) or with PCA with the described technics. About 5 ml of extract is obtained from 1 g of muscle. On the starting line of a sheet of filter paper (Whatman paper No. 1, length about 30 cm; previous washing of the paper with 0.1% Versene (pH 8–9) and water is recommended), apply spots of 0.1 ml of extract, in the form of 10–20 separate drops of 5 to 10 mm^3, which are dried after each application. It is probably preferable first to absorb the nucleotides on charcoal, elute with 2 batches of 50% ethanol of $^1/_5$ of the original solution volume, and thus start with a more concentrated and less contaminated material. Marker spots of pure nucleotides are also placed on the paper.

After drying, the chromatogram is developed with a medium of 6 parts *n*-propanol, 3 parts concentrated ammonia, and 1 part water with 0.002M Versene. The chromatogram is run descendant for 24 hr at room temperature. During this time, AMP migrates

about 10 cm, ADP less, and ATP least. The spots are located by illumination with shortwave ultraviolet light with a Mineralight lamp by which (possibly by reducing the fluorescence of the paper by absorbing the radiation) the nucleotide spots stand out dark violet on a light background. About 0.05 μg are clearly detectable. The spots so located are circled with pencil and cut out. The nucleotides can be extracted with dilute HCl and quantitatively determined by ultraviolet spectrophotometry.

This method has been used for studying the adenylate kinase reaction (63) and for analyses of muscle extracts (65–67).

Two-Way Chromatography of Muscle Constituents (63, 65)

This is a modification of unidimensional chromatography in which the spots are first developed with one solvent in one direction, and then with another solvent in either the same or the opposite direction. The solvents are so chosen that the first effects certain separations while causing little migration of other components which, in turn, are then separated by the second solvent. Having no experience with this procedure, we refer to the original descriptions.

ANALYSIS BY DIFFERENTIAL ENZYMIC-SPECTROPHOTOMETRIC ANALYSES

A general principle of biochemical analysis is to design an enzymic system of which the substance to be determined is one component, and which is so composed that its reaction with other components, under the influence of specific enzymes, finally leads to a change of the absorption coefficient at a specified wave length, usually situated in the ultraviolet. Methods of this nature were enunciated and brought to high perfection by Warburg (68, 69); their development was further stimulated by Kalckar's investigation (75). The principle of these methods can be explained by the following examples.

1) Determination of adenylic acid by adenylate deaminase. A specific enzyme, not known to act upon other substances under the conditions used, catalyzes the deamination of adenylic to inosinic acid. This causes a change in the ultraviolet spectrum, from an absorption maximum at 260 to one at 250 mμ, the greatest change occurring at 265 mμ. Consequently, measurement of the decrease in adsorption of a solution at 265 mμ as a result of the addition of the enzyme is a measure of the amount of AMP.

2) Determination of ATP with glucose, TPN, hexokinase and glucose-6-phosphate dehydrogenase. With an excess of glucose, hexokinase from yeast will cause the formation of glucose-6-phos-

phate in an amount equivalent to the ATP present; this is then oxidized by TPN under the influence of glucose-6-phosphate dehydrogenase from yeast, as a result of which the absorption band of reduced TPN at 340 mμ appears; its intensity is proportional to the amount of ATP.

3) Determination of inosinic acid. By means of 5-nucleotidase from bull semen, inosine monophosphate and other mononucleotides are dephosphorylated. In the presence of phosphate, nucleoside phosphorylase from liver will convert inosine into hypoxanthine (and also guanosine into guanine, but not, for example, adenosine into adenine), after which by a third enzyme, xanthine oxidase from milk, the hypoxanthine is oxidized to uric acid, with a concomitant rise in adsorption at 290 mμ. This example is well suited to illustrate the specificities involved: AMP, which if converted to adenine would interfere with the xanthine oxidase step, is not formed because of the specificity of a preceding enzyme; at the same time, it also shows the possible difficulties for which tests may have to be performed, since ATP inhibits the nucleotidase and thus prevents the detection of inosine monophosphate. It is therefore a rule that, even more than chemical determinations, the applicability of each of these methods to a given problem has to be carefully tested by measuring the recovery of added known amounts of the material to be determined.

Some other methods, while not exactly fitting this category, will nevertheless be mentioned here. One of these, quite similar in principle to those just mentioned except that the reaction product formed is measured by colorimetric (or other) analysis, is the determination of ADP by means of creatine phosphokinase and phosphocreatine, followed by colorimetric measurement of the creatine so formed. This method is being developed in our laboratory but has not yet been fully tested. Another is rather different in principle, in that the substance to be determined acts not stoichiometrically but catalytically. In a method to determine adenine nucleotides (p. 46), Chappell and Perry (31) arrange that in the phosphorylation of glucose with phosphocreatine, creatine phosphokinase and hexokinase, with the unknown but small amount of ATP and/or ADP (and AMP, if myokinase is also added), the reaction velocity is limited by the concentration of the nucleotide. In this elegant fashion, very small amounts can be quantitatively determined, but the method, of course, does not distinguish between the different stages of phosphorylation since these are continuously converted into each other.

The number of enzymic-spectrophotometric methods is already

sizeable and will certainly increase in the future. However, only a limited number will be described, chiefly those that have found sufficient use in the analysis of muscular tissues to justify their recommendation.

Technical aspects of the measurements.—Customarily, these are performed in the Beckman quartz spectrophotometer or similar instruments. Nearly all measurements rest on a change in adsorption at 265, 290 or 340 mμ, and it is noteworthy that the mercury spectrum contains lines of moderate intensities at or near those wave lengths. We were considering the construction of a separate spectrophotometer for kinetic measurements at those wave lengths, isolated from a mercury-arc by interference filters, which might offer advantages in its operation not easily or inexpensively obtained with an instrument designed for work with a continuous spectrum. An apparatus of German make has been announced (Eppendorf Photometer, see [29]) which appears to fulfill these requirements.

The procedures described are for use in the standard Beckman quartz cells with a total volume of about 3 ml. With nearly the same accuracy, cells of the same optical depth (1 cm) but requiring only 1–1.2 ml can be used. Work with even smaller volumes requires special technics, for the description of which the reader is referred to the literature on ultramicrochemical methods (e.g., 70). Some details of the procedure, such as the volumes in which enzymes or other constituents are added, or the order of their addition, are quite arbitrary and may be changed to fit individual preference. In general, the experimental cuvet contains all constituents but one, and its absorption is read against the blank, which is often water. The reaction is then "triggered" with the missing component, frequently an enzyme, and the resulting change in adsorption is followed until completion. For practical reasons it is desirable that the end value be reached in not much longer than 15 min; however, this means that in the first few minutes the rate is so high that the original extinction value cannot be extrapolated with any accuracy. Some investigators attempt this nevertheless by taking a reading 5–10 sec after the last addition. We prefer to evaluate the reading at 0 time by calculation, taking into account *1*) dilution of the sample by the addition of the last reagent and the influence of this upon the original value of the extinction coefficient, and *2*) the light absorption, if any, of the last added reagent itself. If this is an enzyme and the measurements are made at 265 or 290 mμ, the latter effect may be significant.

Materials added in quantities of 0.1 ml or less are best trans-

ferred by constriction pipets, calibrated "to deliver" (see 70). These can be easily made in the laboratory, as their accurate content (e.g., whether 0.057 or 0.0046 ml when about 0.050 is required) is immaterial so long as the same pipet is used for the same reagent throughout a series of measurements. Mixing in the cuvets can be effected by blowing air through the solution after emptying the pipet, or with small stirrers which are so constructed that they can be left in the cuvets without protruding into the light path (this is not feasible in the 1 ml Beckman cells).

Since a series of analyses may take a whole day, it is necessary to keep the working solutions of enzymes and other sensitive materials on ice during the course of the measurements. It is easy to construct a test-tube holder fitting into an insulated container for crushed ice, in which the solutions are kept cold and within easy reach.

The foregoing extensive descriptions of the preparation of enzymes from rabbit muscle were given partly because of their applicability to analytic purposes. Methods for the purification of a few enzymes which are derived from sources other than muscle follow.

PREPARATION OF ENZYMES NOT DERIVED FROM MUSCLE

YEAST HEXOKINASE

This enzyme is used for the determination of ATP with hexokinase and Zwischenferment, and can also be used in the adenylate deaminase scheme of measuring AMP, ADP and ATP. A description of its isolation is unnecessary since a good preparation which can be used for these analyses is commercially available.* It is supplied as a lyophilized powder of enzyme and glucose; 10 mg, to which, if desired, 100 mg of glucose may be added, are dissolved in 10 ml of water, and the solution is used as such. The preparation contains nucleoside diphosphokinase, so that triphosphates other than ATP are included in the analysis. If this is objectionable, further purification of the enzyme is necessary, according to published procedures (71).

ZWISCHENFERMENT (GLUCOSE-6-PHOSPHATE DEHYDROGENASE)

The brief procedure of LePage and Mueller (72) is suitable for most purposes. A more highly purified enzyme is prepared according to Kornberg (73). Suspend dried brewer's yeast† in 3 vol of 0.1M $NaHCO_3$, saturated at room temperature with 5% CO_2 in

* Pabst Laboratories, Milwaukee, Wis.
† Anheuser-Busch, Inc., St. Louis, Mo.

N_2. Keep the suspension for 6 hr at 37° in a large beaker, for autolysis. All further steps are performed in the cold. Centrifuge the suspension, and keep the supernatant solution in the cold overnight. Add to each 100 ml of autolysate, 300 ml of 0.1M $NaHCO_3$ saturated with 5% CO_2, followed by 140.5 g of solid ammonium sulfate. After 15 min of gentle stirring, remove the precipitate by centrifugation and discard. Add to the supernatant solution a further 30 g of ammonium sulfate for each 100 ml of autolysate originally used. Collect the precipitate and dissolve in 500 ml water (roughly to same volume as the original autolysate). Add a suspension of 4 g aged calcium phosphate gel to adsorb the enzyme (15 min stirring in the cold), and centrifuge the gel off. Suspend it in 500 ml of cold 0.1M potassium phosphate buffer, pH 7.5, and stir for 20 min to elute the enzyme; add 168 g of ammonium sulfate to the supernatant solution obtained by centrifugation of the suspension, and remove the precipitate by centrifugation; add 30 g ammonium sulfate to precipitate the enzyme. Collect this by centrifugation, and dissolve in 50 ml water; add 250 ml of 0.1M acetate buffer, pH 4.4; centrifuge the precipitate off after 10 min in the cold and dissolve in 10 ml 0.2M glycylglycine buffer, pH 7.4, and lyophilize. Before use, dissolve 50 mg dry powder in 5 ml water.

APYRASE

This enzyme, which liberates both terminal phosphates from ATP and other triphosphates, and the terminal phosphate from diphosphonucleosides, is prepared according to Nguyen-van Thoai *et al.* (74). Peel 1 kg of potatoes and cut as for the preparation of French fries. Mince in a Waring Blendor or grind in a meat grinder with 600 ml cold 0.01M KCN, pH 7. Cyanide is used to prevent browning of the juice by the action of polyphenol oxidase since the dichinones so formed may inactivate the enzyme; presumably, other enzyme inhibitors, such as azide, may serve as well. After extraction for 30 min, filter the mixture and press through gauze. Adjust the pH to 5.0 with glacial acetic acid, and centrifuge the mixture. Dialyze the supernatant for 1 day against several changes of water. Collect the abundant precipitate so formed by centrifugation, and extract 3 times with 100 ml batches of 0.5M KCl and 0.001M KCN (150 ml may be taken for the first extraction), each time for 15–30 min with stirring in the cold. In the combined extracts, the pH is adjusted to 6.5. The enzyme is now fractionated in the cold with ammonium sulfate, the fraction precipitating between 0.4 and 0.6 sat being collected. Dissolve this in

60 ml of 0.001M KCN and dialyze overnight against KCN solution. The pH is then adjusted to 5.0. Then slowly add cold ethanol (−20°) to a final dilution of 10% (N/N), and discard the precipitate. Add more ethanol to 30% conc. Collect the precipitate, dissolve in 40 ml water, and refractionate with ethanol, the fraction between 15 and 25% being collected; repeat the last step.

ENZYMIC-SPECTROPHOTOMETRIC DETERMINATION OF NUCLEOTIDES

DETERMINATION OF AMP WITH ADENYLATE DEAMINASE (75)

The principle of this method has already been explained.

Reagents.—1) Adenylate deaminase (prepared from muscle, p. 14). *2)* Succinate buffer, 0.2M, pH 6.1.

Procedure.—While the blank cuvet contains water, the experimental one contains 1–2 ml of buffer, the sample, and water to 3 ml final volume. As much sample as possible should be taken, provided that D^{265} does not exceed the measurable range; optimally, with the Beckman spectrophotometer it should read close to 1.0. At 0 time, 0.05 or 0.10 ml of deaminase solution is added, and the D values are read until constant.

Evaluation.—We assume the value of ΔD^{265} relative to the adenylate concentration to be: $\Delta M = 8.0 \times 10^3$; in terms of quantities as measured, at a final concentration of AMP in the cuvet of 0.10 μM/ml, D^{265} would decrease by 0.8 unit.

Determination of ADP in Addition to AMP

The preceding method can be extended to ADP by adding adenylate kinase (myokinase) after the deamination of AMP has reached completion:

$$\text{AMP} \xrightarrow{\text{deaminase}} \text{IMP} \qquad (1)$$

$$2\,\text{ADP} \underset{\longleftarrow}{\overset{\text{myokinase}}{\longrightarrow}} \text{AMP} + \text{ATP} \qquad (2)$$

The second reaction is an equilibrium reaction, but so long as AMP is removed by deamination it will go to near completion; even so, only half of the ADP is converted to AMP and finally to IMP.

Reagents.—1) Same reagents as for AMP, but the succinate buffer should contain 0.002M $MgCl_2$, or $MgCl_2$ is added separately to a final concentration of 0.001M or higher in the system. *2)* Adenylate kinase (myokinase) from muscle (p. 15).

Procedure.—The AMP assay is performed as already described.

After its completion, 0.05 ml myokinase is added, and the reaction is again allowed to proceed.

Evaluation.—Since only half of the ADP present eventually appears as IMP, the sensitivity for ADP is half as great as for AMP; hence, at a concentration of 0.1 μM ml in the final system, $\Delta^{265} = 0.4$. Due to this decreased sensitivity, and the circumstance that the reaction may not quite reach completion within a practicable time, this method of determining ADP is not ideal under all circumstances. Its great simplicity, however, warrants its use before resorting to any of the more complex procedures.

Determination of ATP in Addition to AMP and ADP

After the preceding two tests have been carried out, ATP can finally be determined by its conversion into AMP, either directly by apyrase, or with glucose and hexokinase to ADP, which is further dephosphorylated by the myokinase still present. Hence, the reactions already formulated are followed by:

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{ADP} + \text{glucose} \qquad (3)$$

or:

$$\text{ATP} \xrightarrow{\text{apyrase}} \text{AMP} + 2\text{P} \qquad (4)$$

Reagents.—*1)* Same reagents as for ADP, except that a somewhat higher Mg concentration can be used for the hexokinase. *2)* Potato apyrase (p. 41) or hexokinase containing glucose (p. 40).

Procedure.—After completion of the AMP and ADP assays, add the additional enzyme and read D^{265} until it reaches constancy. When working with extracts of freshly dissected muscle, it will be seen that this usually contains mainly ATP, no AMP, and some ADP just at the limit of detectability.

Evaluation.—Since all ATP is converted into AMP, we have again $\Delta^{265} = 0.80$ when 0.10 μM ml are present. However, the ATP reading also includes half the ADP, which therefore has to be subtracted.

The three methods just described are ultimately based upon the deamination of AMP, so that the specificity of this system of AMP–ADP–ATP analysis is very great. Some complications may arise from phosphate-transferring enzymes such as nucleoside diphosphokinase; however, these can be removed from the system (least easily, perhaps, when hexokinase rather than apyrase is used). Apart from the lower accuracy of the ADP step, and the frequent sluggishness in reaching the end point, the method can be highly recommended. It has been used for the analysis of cardiac and skeletal muscle extracts (76, 77, 34).

Other Procedures Involving Adenylate Deaminase

It appears likely that, in conjunction with specific phosphotransferases now being discovered (78), adenylate deaminase may become useful for the determination of certain nucleoside diphosphates. However, this must await further developments. A specific deaminase for ADP (64) may find analytic application.

METHODS BASED ON OXIDATION OF HYPOXANTHINE AND XANTHINE TO URIC ACID

As was mentioned before, hypoxanthine can be determined by the optical change accompanying its oxidation, via xanthine, to uric acid by xanthine oxidase. This enzyme will also very slowly oxidize adenine, and we have found that the oxidation of hypoxanthine is inhibited by adenine, so that the analysis of mixtures is not possible. However, the determination of inosine (e.g., as a nucleotide breakdown product in anaerobiosis) in conjunction with nucleoside phosphorylase would be a useful application, and several other uses can be foreseen. As explained before, the system cannot be used to determine IMP in the presence of ATP in muscle extracts. In view of the limited significance of these methods so far, we shall not discuss them further.

SYSTEMS BASED ON OXIDATION OR REDUCTION OF DPN OR TPN

Several excellent methods of analysis depend on coupling with a reaction leading to the reduction of DPN or TPN, or to an oxidation of the reduced form of these coenzymes. Only a few which have been found useful in work on muscle will be described, and a few others will be outlined briefly. The development of many more applications is likely, as pure enzymes for given substrates become available.

Since quite pure preparations of DPN and TPN are now available at reasonable prices, we will not discuss their preparation or purification. Like other labile factors, they are stored dry at low temperature. Small amounts of working solutions are kept frozen for a limited time.

Reduction of DPN

It can be carried out chemically or enzymically. The chemical method is convenient and will be described (79, 80). Place 10 mg of DPN, in a 13 × 100 mm test tube, add 2 ml of a fresh solution of 0.2% sodium hydrosulfite in 1% $NaHCO_3$, and immerse the tube immediately in boiling water for 1 min; the yellow color should fade within 30 sec. Cool the tube, add 6 ml cold absolute ethanol, and

keep the mixture at $-10°$ C for 20 min. Then centrifuge at that temperature to remove precipitated salt. Add the supernatant solution to 24 ml cold absolute ethanol, and centrifuge the tubes at that temperature. Decant the supernatant solutions and dry the precipitates *in vacuo* at $-10°$. The content of each tube, when dissolved in 1.5 ml water, will yield a solution containing about 1 μM/ml. The dry material can be kept, even at low temperature, for a limited time only. Storage in an inert gas may be advantageous.

Determination of ATP in Terms of TPN Reduction (73)

The principle of this method has already been explained. The following reactions are involved:

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{ADP} + \text{glucose-6-phosphate} \qquad (5)$$

$$\text{glucose-6-phosphate} + \text{TPN} \xrightarrow{\text{dehydrogenase}} \text{phosphogluconic acid} + \text{TPN-H} \qquad (6)$$

Reagents.—*1*) Tris(hydroxymethyl)aminomethane buffer or glycylglycine buffer, 0.2M, pH 7.6, containing 0.01M $MgCl_2$. *2*) Hexokinase-glucose solution: 10 mg commercial hexokinase and 100 mg glucose dissolved in 10 ml water. *3*) Glucose-6-phosphate dehydrogenase (Zwischenferment) from yeast (p. 40), 10 mg dry powder dissolved in 1 ml water. *4*) TPN, commercial preparation of 80% purity or better, 30 mg in 5 ml water.

Procedure.— In a 3 ml cuvet mix 2 2.4 ml buffer, 0.4–0.8 ml sample (diluted, if necessary), 0.1 ml Zwischenferment and 0.05 ml hexokinase-glucose solution. Measure the absorption at 340 mμ. The reaction is initiated with 0.05 ml of TPN solution, and the extinction coefficient is read until constancy.

Evaluation.—Since the change in D^{340} accompanying the reduction of TPN amounts to 6.22 for a 10^{-3} M solution, a Δ^{340} of 0.194 corresponds to the presence of 0.1 μM of ATP in 3.2 ml of solution in the cuvet. The method works well with muscle extracts. However, with the usual enzyme preparations as employed for these analyses, the myokinase especially is likely to contain nucleoside diphosphokinase, so that the method is not specific for ATP, but for nucleoside triphosphates in general. With pure enzymes, the method is specific, so far as known substrates are concerned.

Determination of ADP with Reduced DPN (22)

This method is based upon the reactions:

$$\text{ADP} + \text{phosphopyruvate} \xrightarrow{\text{kinase}} \text{ATP} + \text{pyruvate} \qquad (7)$$

$$\text{pyruvate} + \text{DPN-H} \xrightarrow{\text{lactic dehydrogenase}} \text{lactate} + \text{DPN} \qquad (8)$$

Reagents.—1) Phosphopyruvate, K salt, 0.005M (p. 32). *2)* 0.1M $MgCl_2$. *3)* 0.001M reduced DPN solution (p. 44). *4)* Phosphate buffer, 0.5M, pH 7.4. *5)* Lactic dehydrogenase from muscle (pp. 18, 20), diluted 20- to 40-fold. *6)* Pyruvate phosphokinase from muscle (pp. 16, 21), diluted 20- to 50-fold.

Procedure.—In a 3 ml quartz cuvet mix 0.1 ml phosphopyruvate, 0.1 ml of $MgCl_2$, 0.2 ml or more of reduced DPN, 0.2 ml of phosphate buffer, and 0.05 ml pyruvate kinase, with water to a volume of 2.95 ml. The reaction is started with 0.05 ml lactic dehydrogenase, and is followed until completion. The pyruvate kinase can be omitted at first, to test for the presence of free pyruvate, and the ADP determination then run separately by adding pyruvate kinase.

Evaluation.—As in other DPN or TPN tests, a change in optical density at 340 mμ of 0.622 corresponds to a concentration of 0.1 μM/ml, or a Δ^{340} of 0.207 at a total volume of 3 ml corresponds to 0.1 μM in total. In this case, unlike the foregoing one, the initial absorption is sizable, and decreases during the measurement.

The method works well with muscle extracts (34). Its specificity has not been completely described, and a few diphosphates other than ADP may be included in the determination.

DETERMINATION OF ADENINE NUCLEOTIDES WITH HEXOKINASE AND CREATINE PHOSPHOKINASE (31)

This elegant method provides a highly sensitive (below 0.01 μM) method for the determination of ADP and/or ATP, without distinguishing between the two. The following reactions are involved:

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{ADP} + \text{glucose-6-phosphate} \qquad (9)$$

$$\text{PC} + \text{ADP} \xrightarrow{\text{creatine kinase}} \text{ATP} + \text{creatine} \qquad (10)$$

$$\text{AMP} + \text{ATP} \xrightarrow{\text{myokinase}} \text{ADP} \qquad (11)$$

Reaction (*11*) can be brought into play when myokinase is added as well, and AMP would then be included in the analysis; otherwise, only reactions (*9*) and (*10*) are dealt with. In summary, these result in the phosphorylation of glucose at the expense of phosphocreatine, at a rate determined by the amount of ADP or ATP added. This rate is measured by colorimetric determination of the creatine formed. Since this is not a method for measuring total change but for measuring rate of change, procedures permitting

continuous measurement would have some practical advantage; coupling with an excess of Zwischenferment and TPN and measuring the rate of TPN reduction suggests itself as one procedure. For full details, see the original report (22).

DETERMINATION OF ATP BY BIOLUMINESCENCE (81)

Since ATP can elicit light production in firefly extracts, and the measured light intensity is proportional to the amount of ATP, a method has been developed to determine ATP, as well as substances which indirectly can supply ATP by coupled reactions with specific enzymes. One of the advantages would be in the great sensitivity which can be obtained with suitable apparatus. However, not enough experience has been gained in the use of the method with tissue extracts to judge its reliability and specificity.

DETERMINATION OF ATP BY ITS EFFECT ON ACTOMYOSIN (82)

Addition of ATP to a solution of actomyosin causes a physical change in this protein, evident from a reduced viscosity or turbidity. The change is transient, enzymic dephosphorylation of the ATP causing a reversal toward the original condition. The magnitude of the original response or the duration of the response (83), which is more accessible to measurement, can be used as a measure of the amount of ATP, by calibration with known amounts of ATP. The method requires further standardization before it can be recommended.

ENZYMIC DETERMINATION OF SUBSTRATES

Obviously, the possibilities of differential enzymic spectrophotometry are not restricted to nucleotides, but can be extended to all other substances which may be involved in reactions similar to those already described. Several such methods have indeed been worked out (see 84), but most of them have not yet been tested for their applicability to the analysis of extracts of contractile tissues and will therefore not be discussed here.

III. SOME METHODS OF EXPERIMENTATION WITH COMPLEX SYSTEMS

These methods differ from those described in the preceding sections in that they refer not to methods of analysis or of preparation of individual substances, but to more complex investigative procedures. As these often constitute the characteristic experimental approach of a given investigation, it will not be feasible to give a wide selection from such material here. On the contrary, the experimental design is such an essential part of an investigation that it is inadvisable to sever it from the results obtained and the conclusions derived. We make exception, however, for the measurement of ATPase activity and the manufacture of contractile fiber preparations.

Some other complex experimental procedures play an important role in the investigation of muscle proteins. Some of these have already found place in earlier volumes of this series, e.g., ultracentrifugation and electrophoresis. For muscle proteins, the latter method has been described extensively by Dubuisson (5, 6). Flow birefringence, a method which has played such a great role in work on myosin and actin, has also been described (87).

MEASUREMENT OF ADENOSINETRIPHOSPHATASE ACTIVITY

GENERAL METHOD

Most of the work on myosin ATPase and other ATPase systems has been done by determining the inorganic phosphate liberated during the reaction, usually by the Fiske–SubbaRow procedure. No further description of this is necessary, except to state that, as in other enzyme studies, the course of the reaction must be determined by analyses at several time intervals. Conclusions based on a single determination after a given time may be grossly misleading if the reaction velocity changes considerably during the run.

ELECTROTITRIMETRIC METHOD

For the study of enzyme kinetics particularly, it would be of great advantage to determine the course of the reaction continuously or semicontinuously by a measurement which can be carried out repeatedly during the reaction in the same sample. The best example of this is given by the optical measurements of the type described for differential enzymic spectrophotometry (pp. 37–47). However, the splitting of ATP to ADP is not accom-

panied by any optical change. It is possible, of course, to couple the ATPase with other enzymes which cause an optical change indirectly. For example, a mixture of phosphopyruvate, reduced DPN, pyruvate phosphokinase and lactic dehydrogenase could be incubated with the ATPase. In this manner, the splitting of ATP would eventually express itself as a reduced extinction at 340 mμ. However, we would then be dealing not with the kinetics of a single enzyme but of a chain of enzymes, and only under favorable circumstances could the observed phenomena be unequivocally ascribed to the ATPase itself. In this particular case, the intrinsic activity of the additional enzymes is very high, so that a careful study of this possibility may be well worth the effort (cf. 88), but has not yet been carried out.

The electrotitrimetric assay of ATPase is based on the fact that acid is liberated in the splitting of ATP to ADP (cf. 89). This effect has been utilized to demonstrate the presence of ATPase by noting the drop in pH (90, 91) or measuring the CO_2 liberated from a bicarbonate buffer (90). The disadvantage of the latter procedure is that the span of available pH values is limited to the range of bicarbonate–CO_2 buffers; in the former, the pH changes during the measurement. In the electrotitrimetric procedure, the pH is kept constant throughout the reaction by adding small increments of standardized KOH; the rate of KOH consumption is a direct measure of the rate of decomposition of ATP.

In most other cases in which the electrotitrimetric method had previously been successful, e.g., in the splitting of fats or esters be lipase, or in the assay of cholinesterase, the situation is simple in that 1 equivalent of base is consumed for each mole of substrate decomposed. This is not so in the case of the ATP–ADP reaction, and a special investigation was required to test the applicability (92).

The first feature is that the amount of acid liberated (base consumed) per mole of ATP depends on the pH. Above pH 8, this ratio is unity until at much higher pH a further change occurs, with which in practice one will hardly ever be dealing. At lower pH, the ratio decreases according to what we have termed the *equivalence curve*; the ratio, equivalents of acid produced per mole ATP split, is called the *equivalence ratio*. The equivalence curve, furthermore, depends strongly on the electrolyte composition of the medium, in particular on the concentration of divalent cations, which form complexes with the substrate and its split products. However, the equivalence ratio does not change during the course of the reaction and does not depend on the substrate concentration, except for possible complications due to complex formation with

bivalent cations. These interactions are fully described in the original paper. The following summary may be useful in planning the use of the method. *1*) Above pH 8, the equivalence ratio is unity; hence, the consumption of 1 ml of 0.01N KOH indicates the splitting of 10 μM of ATP. *2*) At a pH near 6.9 (determined as pH 6.88 in our work), the equivalence ratio is 0.5 in all media investigated. *3*) At pH values well below 6.9, the equivalence ratio becomes small and is quite sensitive to pH and salt composition, so that special precautions are necessary.

Procedure

The method can be used under a variety of conditions with respect to volume of sample, concentration, etc. In the original procedure (92), a 50 ml beaker is kept in a water bath at constant temperature, and will hold 25 ml of reaction mixture. This is stirred by a glass stirrer driven from above, and a slow stream of CO_2-free air or nitrogen is passed over the surface of the solution; this may be superfluous at sufficiently low pH. The vessel further contains a glass electrode and a calomel electrode connected with a pH meter, which allows reliable measurement of the first, and estimation of the second, decimal point. The vessel finally holds the tip of a sensitive buret (conveniently a horizontal type of microburet) which allows 0.1 ml additions to be made accurately. The vessel contains the entire reaction mixture, except for the enzyme, and is preincubated at the temperature and pH of the experiment. The enzyme adjusted to the same pH is in a small container at the same temperature, and is added in small volume from a micropipet, calibrated "to contain" to initiate the run. Before adding the enzyme, the pH is observed for some time, to test its constancy. A timer is started when the enzyme is added, and a small amount of alkali is added when, as a result of the enzymic reaction, the pH has dropped somewhat. With some experience, one will be able to let the pH fluctuate, between additions, within narrow limits. After the addition of 0.1 ml of 0.01N KOH, for example, the time is noted after which the original pH is again reached (the pH setting of the meter is not changed, but a watch is kept for when the needle or indicator passes the zero position). After a sufficient number of such readings, the experiment is finished. The data so obtained give a plot indicating the liberation of acid as a function of time, and when the equivalence ratio is known, yield the splitting of ATP as a function of time.

For favorable cases (pH 8 or above; pH 6.88) the equivalence ratio has just been given. For the pH 6.9–8.0 range, it can be read with fair accuracy from the figures given by Green and Mom-

maerts (92), except that the possible dependence of the Ca and Mg effects upon the substrate concentration has not been investigated. For all doubtful cases, determination of the equivalence ratio during the experiment is recommended. This can be done either by measuring the splitting of an accurately known amount of substrate to completion, or by measuring the formation of inorganic phosphate during a certain period in the reaction mixture.

Several applications of the method have been reported (93-95), and an apparatus for microdeterminations has been described (96).

OTHER ELECTROTITRIMETRIC ASSAYS

There are several other important muscle enzymes which could be investigated electrotitrimetrically. For example, base is liberated in the formation of ATP and creatine from ADP and PC; several other possibilities could be listed. In each case, extensive calibration work will have to be performed, since again complicated ionic effects are to be expected. Such work will be rewarding only when kinetic investigations are envisaged.

GLYCEROL-EXTRACTED MUSCLE PREPARATION

The historic significance of the discovery of the contractile actomyosin thread by Szent-Györgyi in 1942 does not need to be recalled at this point (see 101, 106). Several actomyosin preparations have since been described which constitute a technical improvement over the earlier one; these have been obtained by controlled drying of actomyosin threads (97), or by folding surface-spread actomyosin films (98, 99). With the latter procedure, Engel'gardt (100) even obtained contractile myosin filaments without actin.

We shall describe, in detail, another type of preparation, likewise originated by Szent-Györgyi (101); the glycerol-extracted fiber bundle made from the psoas muscle of the rabbit. Some other muscles may be used as well, and the long neck-retracting muscles of turtles are said to be a favorable material (102). Similar preparations have been obtained from heart (103) and from smooth muscle (104).

A rabbit is dissected after anesthesia with $MgSO_4$ or other narcotic, or after curarization, as described earlier. The viscera and the body wall are removed so as to expose the psoas muscle, and the whole is thoroughly cooled in ice. By blunt dissection, 1–2 mm thick bundles of muscle fibers of the muscle are separated, and are tied, at about the rest length, with soft cotton thread to wooden applicator sticks. They are then detached, and immediately placed in 50% (by volume) of glycerol with a small amount of bicarbonate, at 0°. The tubes of glycerol are kept in ice in a Dewar flask placed

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in the cold room, and the fibers are then transferred to a fresh tube of 50% glycerol and stored at −20° C.

Before use, a stick with adherent fibers is transferred to a tube with cold 10% glycerol, and slowly warmed to room temperature. The material is then detached from the stick and placed horizontally in a shallow tray with 10% glycerol on a black background, and illuminated from the side. With fine tweezers, the strip is split longitudinally into small fiber bundles, 0.1–0.2 mm in diameter or thinner. Fibers 2–3 cm long are folded to a loop and tied on one end with a string of yarn. To investigate the contractile response, the loop is attached over a hook of the measuring apparatus, and the string is attached to the recording arm of the measuring apparatus.

The contraction is elicited by ATP in a suitable medium, e.g., 0.16M KCl with 0.01M $MgCl_2$, and can be recorded isotonically or isometrically. In either case, a low-friction recording system has to be used, since the actual forces developed by thin fiber bundles are small. In 1953, a method was developed for isometric recording with the aid of an RCA 5734 mechanical-electrical transducer and an Esterline-Angus paper-writing galvanometer, later replaced by a Recti-riter,* which has similar characteristics of speed and sensitivity, but has the advantage of rectilinear instead of curvilinear co-ordinates. This assembly, which is quite comparable to the one independently designed by Ranney (105), will be described below by Schilling.

The responses of the glycerol-extracted fiber and their dependence on the ionic composition of the medium are quite variable, according to the presence or absence in the preparation of the so-called relaxation factor. The rather extensive literature on this subject has been reviewed elsewhere (106, 107).

Appendix

AN ISOMETRIC TENSION RECORDER

by MYRON O. SCHILLING

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Time-tension curves are recorded on the linear chart-recorder R, which is a Recti-riter,* or other recording device. The output signal from the RCA 5734 transducer is amplified by the tensiometer circuit as shown in Figure 1. A sensitivity control and a zero adjustment are provided. Voltage regulator tubes provide adequate

* Manufactured by Texas Instruments Inc., Houston 6, Texas.

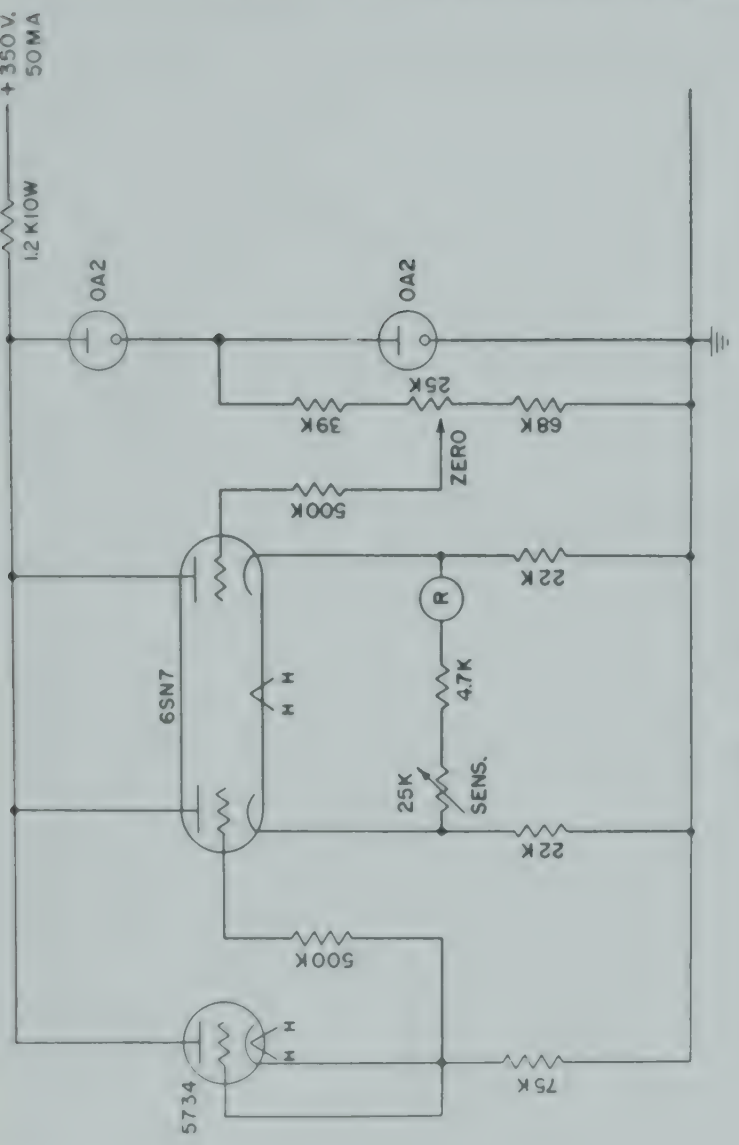

Fig. 1.—Tensiometer circuit.

plate voltage regulation, but, to reduce any drift, it is recommended that the heater voltage also be stabilized by a 1% = constancy line stabilizer. The transducer stylus has been extended by a length of hypodermic needle tubing, such that one gram of tension produces more than full deflection of the recorder. Stops are provided to limit deflections in all planes to $\pm 1/2$ degree to avoid damage to the transducer. By employing the stylus in vertical direction, the advantage is gained of having no deflection for zero tension. This necessitates placing the muscle horizontally, which somewhat limits the possible experimentation, but is perfectly

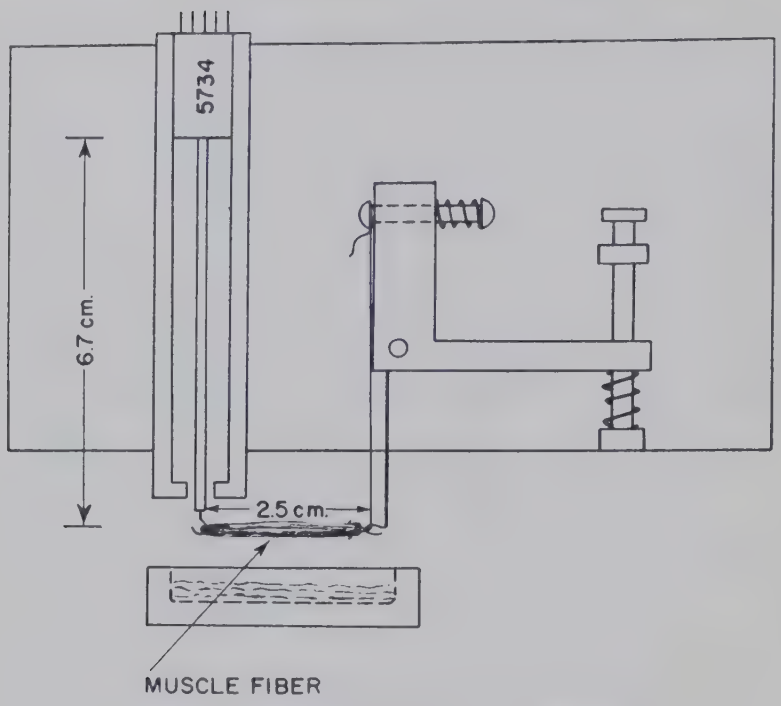

Fig. 2.—Transducer and muscle assembly.

satisfactory for work at room temperature without regulation. In this case, as is shown in Figure 2, the muscle rests in fluid in a chamber machined from Lucite, and is strung between the transducer stylus and an adjustable clamp. Changes of fluid are made by changing individual chambers, or by means of a fine hypodermic needle inserted through a hole drilled through the wall of the chamber. If desired, it is not difficult to invert the apparatus for use with a vertically placed fiber.

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SECTION II

Hemodynamic Methods—Heart and Lungs

ASSOCIATE EDITORS
Richard Gorlin and *James V. Warren*

INTRODUCTION

SINCE THE FIRST VOLUME in this series was published almost ten years ago, there have been many advances in the field of cardiovascular physiology. Methods of study have been developed which permit the investigator to measure functions in man that were heretofore susceptible to study only in the experimental animal. As a result, a new body of information is developing on human cardiovascular physiology.

Determinations of cardiac output in man are at present carried out mainly either by the catheter method, utilizing oxygen consumption data obtained by the direct Fick principle, or by the dye injection technic of Hamilton and Stewart. The former method was discussed in Volume I, and the reader is referred to it for basic orientation on the catheter procedure itself. As yet, most investigators rely on oxygen data alone, the analyses on blood being carried out in many laboratories spectrophotometrically rather than by blood gas analysis by Van Slyke's technic. The oxygen consumption is measured in different ways in various laboratories. The most frequent method is probably the collection of a sample of expired air with analysis of at least oxygen, and in some instances, oxygen and carbon dioxide by means of a paramagnetic apparatus. In some instances, determination by the Haldane apparatus or one of its derivatives is still utilized, in others a metabolism apparatus is chosen. Although there are considerable minor technical variations, the method remains essentially as described in 1948.

Since then, the dye injection technic has gained considerably in popularity. The basic mechanism involved is presented in this volume. There are at present many variations, utilizing substances other than Evans blue dye (T-1824). These include other dyes, inorganic compounds and radioactive material. Because of space limitations, we were unable to include all these methods and the additional variants in calculation, but references are given for some of the more frequently used technics.

Other methods frequently encountered by the student of human cardiovascular physiology are included. These represent only a small selection of a widespread development of methodology in this field during recent years.

—Richard Gorlin and James V. Warren

DYE METHOD FOR DETERMINING CARDIAC OUTPUT

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BACKGROUND

THE METHOD IS based on the principle that if a marking substance is injected into a flowing liquid the degree of dilution after adequate mixing is a measure of the volume of flow. In a closed system, the measurement must be made during the first circulation of the injected substance. The principle was first employed by Stewart in 1897 (6), and later elaborated by him (7, 8). Hamilton and co-workers (3) revived the method 30 years later, and extended it by using nondiffusing, protein-binding dyes. The validity of the principle was established in glass models and in the heart–lung preparation. In the anesthetized dog, a close correlation was found between the cardiac output measured by the dye dilution and by the Fick methods. When applied to human beings, the method yielded values for the cardiac output regarded by contemporary critics as too high in comparison with those obtained by the then popular Grollman acetylene technic. As a consequence, the dye method fell into disrepute. When, 10 years later, the direct Fick method of estimating blood flow by venous catheterization of the heart became applicable in human beings, the values for the cardiac output derived by it were considerably higher than those found by the acetylene method. In 1948, Hamilton, Cournand, and associates (4) reported the results of a study showing satisfactory agreement between the direct Fick and the dye methods. The dilution method has since been extensively employed by various groups in this country and abroad. Dow's (1) excellent review was published in 1956.

The product of the blood flow and the mean circulation time gives the volume of blood between the point of injection of the marking substances and the point of collection. This measurement, termed "Q" by Stewart, comprises the vascular territory between the point of injection in an arm vein and the point of arterial collection, and thus may be regarded as a crude index of the intrathoracic blood volume. In 1949, Ebert and co-workers (2) proposed a refinement wherein the dye is injected directly into a pulmonary artery through a cardiac catheter rather than into a peripheral vein, as in Hamilton's method. It is thereby possible to measure the mean circulation time of the dye between the pulmonary artery

and the point of arterial collection. The blood flowing during this period can be measured by the direct Fick, or, preferably, by the dye dilution method.

Only the basic procedure is presented here. It has the great advantage over the more elegant modifications of initial technical simplicity. The advantages of the specific variations are discussed by Dow (1).

Clinical application of the mathematical and physical significance of the contour of indicator dilution curves has been studied by Korner and Shillingford (5). They demonstrated that the steepness of the down slope of the curve was related directly to cardiac output, and inversely to blood volume between sites of injection and sampling and valvular regurgitation. By determining the first two variables and using an empirically derived equation, it is now possible to apply indicator dilution curves to quantitative valvular incompetence.

LABORATORY EQUIPMENT AND PROCEDURE

EQUIPMENT

1) 1 cc Wintrobe hematocrit tubes. *2*) 4 cc pointed centrifuge tubes. *3*) 12 or 15 cc centrifuge tubes. *4*) Centrifuge multiple carriers and rubber cushions to hold 4 cc tubes; International #381, $\frac{1}{2}$ inch diameter holes. *5*) Drifilm (or SC-77).* *6*) Thin glass rodding (4 mm diameter) pulled to a fine point. *7*) Beckman spectrophotometer equipment—Corex 23-10-73 or Silica 23-10-89 absorption cells (also used with O_2 method). *8*) Fused silica micro cells 3 × 10 × 25 mm.† *9*) Cell carrier. *10*) Cell carriage holder. *11*) Diaphragm attachment. *12*) 3 cycle semilog paper—6 divisions/inch (K & E 359-70). *13*) Calibrated syringes. *14*) Calibrated catheters. *15*) Siliconed glassware.

Calibrated Syringes

5 cc. syringes are marked on the barrel and plunger with a diamond point so that an exact setting can be made. The following marking is convenient: —.— The lines on the barrel should be perpendicular to the lines on the plunger and the points set together. 2 and 3 cc volumes are usually used.

Since the syringe is not washed before dye output collection and is washed before the blood volume samples are collected, it is necessary to calibrate syringes "to contain" and "to deliver." This can be done by weighing the marked syringe and cap (taped to a tongue depressor if necessary to prevent rolling). Draw water or

* General Electric Company, Silicone Products Division, Waterford, N. Y.
† Pyrocell Manufacturing Co., 207 E. 84 St., New York 28, N. Y.

dye into syringe, remove bubbles, adjust to mark, cap, and weigh. The difference in the 2 weights converted to cc is the volume contained. Then shoot the water into a weighed container and weigh the container and water. The weight difference converted to cc is the volume delivered. Repeat until volumes agree to 0.01 cc.

Calibrated Catheters

For cardiac output determinations, when the dye is injected through a catheter the volume of the catheter has to be subtracted from the volume the syringe delivers. The catheter is flushed before the dye volume samples are collected and therefore needs to be calibrated "to contain" only. This can be done with mercury, using a 3 way stopcock. It is necessary to know the stopcock volume from the cutoff to the end connected to the catheter: fill the end to be calibrated with mercury, empty into a weighed container, and weigh; convert the weight difference to cc; repeat until volumes agree to 0.01 cc.

Connect a syringe containing mercury to the stopcock, and connect the catheter to the calibrated end. Stretch the catheter out on a level surface. Push the mercury through the catheter until a drop appears at its end. Turn the stopcock so that the catheter end is closed. Put the tip of the catheter into a weighed flask, disconnect the syringe, shake all the mercury out of uncalibrated ends of the stopcock, open the stopcock and let the mercury fall into the flask. Connect the syringe to the stopcock, push air through the system to get out all the mercury, and weigh the flask and mercury. Repeat until weight differences converted to cc agree to 0.01 cc. Subtract stopcock calibration.

Siliconed Glassware

It is recommended that tubes used for blood samples be siliconed with Drifilm. It can easily be sprayed on the tubes if a hood is available. Clean, thoroughly dry tubes are arranged in a box. A suction flask or bottle fitted with a 2-holed stopper is arranged so that it may be connected to an air line, and a piece of glass tubing (approx. 10 inch) is connected to the flask or bottle. A few cc of Drifilm are poured into the flask. The air is cut on and the glass rod is moved slowly over the tubes. The vapor settles and covers even the tips of the tubes. Leave the air on long enough to vaporize all of the Drifilm, and blow air over the tubes; or allow tubes to stand 15 min before washing. Wash tubes in water, rinse with distilled water, and dry. Since Drifilm is in strong HCl, protect all metal surfaces. The silicone layer is removed from dirty tubes by soaking overnight in a solution of 10% NaOH in 20% ethyl alco-

hol. This solution may be used many times. Rinse glassware well and dry before reapplying Drifilm.

If a hood is not available, a solution of 1 cc Drifilm in 9 cc carbon tetrachloride can be poured from tube to tube. The silicone can be removed with carbon tetrachloride. Shake the last drop out of the tips of the tubes to prevent a rough thick layer, and allow tubes to dry before washing.

PROCEDURE

Hematocrits.—Cover tubes, spin 30 min at 3,000 rpm. Read and record percentage of packed red cells over total volume.

Hemoglobin.—Pipet 1 cc heparinized blood into a 100 cc volumetric flask partly filled with water. Dilute to mark and read on spectrophotometer at 540 mμ and 620 mμ in 10 mm light path cuvets. Distilled water is used as a blank. Record ratio of 540 mμ to 620 mμ reading. This factor is used later in correcting dye readings for hemolysis.

Blank, Dye Tubes and 10 Min Sample.—Allow to stand at least 1 hr. Loosen any blood or serum clots with a glass rod pulled to a fine point. Centrifuge 15 min at approximately 2,500 rpm. Occasionally clots have to be reloosened and the tubes recentrifuged.

By inspection, determine the tube with the most dye. Select the tubes before the darkest one which seems to have dye and 5 or more tubes after the darkest. Remove serum from these tubes with capillary pipets and place in the silica micro cells. With diaphragm attachment in place so that the medium sized hole regulates the light, read the serum samples against the blank serum. If the blank is hemolyzed, serum from one of the first tubes can be used, provided no dye is present.

The samples are read at 620 mμ and 540 mμ. It is sometimes useful to plot roughly the 620 mμ readings against the time of collection on semilog paper to determine whether a complete curve has been read. Recirculation should be seen on the curve. Read any other samples that will help in plotting the curve. Read the blood volume samples in the same manner.

Cuvets can be cleaned easily between samples by rinsing with saline, distilled water, alcohol and acetone.

CALCULATIONS

CORRECTION FOR HEMOLYSIS

If the 540 mμ reading of the dye sample is 50% or more of the 620 mμ reading, the following calculation can be made. This formula corrects for redness but not for cloudiness of the sample, which also may give a high reading. If the 540 mμ reading is not

greater than the 620 mμ reading, the correction will usually change the 620 mμ reading enough to make a smooth dye curve.

$$\text{Corrected 620 m}\mu\text{ reading} = \frac{(A \times \text{620 m}\mu\text{ reading}) - \text{540 m}\mu\text{ reading}}{A - B}$$

where A = the ratio of the 540 mμ: 620 mμ reading of 1 cc of the patient's blood, and B = the ratio of the 540 mμ: 620 mμ reading of Evans blue dye.

B is determined for each dye lot in the following manner: Several dilutions of dye are made and are read at 620 mμ and 540 mμ on the spectrophotometer; 1:500, 1:1,000, and 1:2,500 dilutions give a good range of readings. The ratio of the 540 mμ:620 mμ reading is calculated for each dilution, and will be approximately 0.5. The ratios should check in the first decimal place.

CARDIAC OUTPUT

Plot the corrected 620 mμ readings against the time of collection on semilog paper. Readings less than 0.010 are usually not plotted. For samples taken at 2 sec intervals, halfway through the interval plus 0.5 sec is used for plotting time (t). Timing for sample collections is begun halfway through the injection, which takes 1 sec. *Example:* Tube 1 is collected at 5–7 sec, tube 2 at 7–9 sec. The 620 mμ reading for tube 1 is plotted at 6.5 sec, tube 2 at 8.5 sec.

Draw the upslope. The downslope is extrapolated as a straight line to the x axis. Record the density readings at each second on the graph.

The sum of the densities at each second is found (ϵc). The density at each second is multiplied by the time and the sum of these products is found (ϵct). The mean circulation time (MCT) or time at which half of the dye has circulated past the collection point is found by the formula:

$$MCT\ (\text{sec}) = \frac{\epsilon ct}{\epsilon c}$$

The concentration by:

$$\frac{\text{mg} \times \text{sec of dye in circulation}}{\text{liter}} = \epsilon c \times EC \times 10$$

EC being the extinction coefficient in terms of mg/100 ml.

The plasma flow (l/min) by:

$$\frac{\text{ml dye injected} \times \text{conc dye (mg/ml)} \times \text{60 sec/min}}{\frac{\text{mg} \times \text{sec}}{\text{liter}}}$$

The volume of dye injected is determined from the syringe volume (calibrated "to deliver") minus the catheter or needle volume. #18 Cournand type needles have been found to contain 0.05 ml. Catheters must be calibrated individually.

The cardiac output is found by the formula:

$$\frac{\text{plasma flow (ml/min)}}{100 - \text{hematocrit}} \times 100 = \text{blood flow (l/min)}$$

The cardiac index by:

$$\frac{\text{cardiac output (ml/min)}}{BSA\ (\text{m}^2) \times 1{,}000} = \text{blood flow (l/min/m}^2\ \text{BSA)}$$

Body surface area (*BSA*) in m^2 is found on the DuBois body surface area chart, when the height and weight are known.

PLASMA VOLUME

The plasma volume (in cc), subject to the errors of the dye method, is found by the following formula:

$$\frac{\text{ml dye injected} \times \text{conc dye (5 mg/ml)} \times 100}{\text{density of 10 min sample} \times \text{EC}}$$

The syringe must be washed into the catheter or needle immediately after the cardiac output sampling, and the needle or catheter must be flushed several times. The amount of dye injected is the total volume the syringe contains.

The blood volume by:

$$\frac{\text{plasma vol (ml)}}{100 - \text{hematocrit}} \times 100$$

It may also be expressed in ml/kg body weight and ml/m^2 BSA.

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NORMAL VARIATIONS IN VENOUS OXYGEN CONTENT

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THE DIAGNOSIS OF left-to-right intracardiac shunt depends on finding differences in oxygen content between two of the cardiac chambers, i.e., dilution of a distal chamber's venous blood with oxygenated blood. It should be appreciated, however, that due to streamline flow, poor mixing and other factors there are rather large variations in venous oxygen content within the compartments of a normal heart. These variations diminish, the more distal the chamber and therefore as more complete mixing occurs. Normal findings are shown in Table 2.

TABLE 2.—NORMAL VARIATIONS IN OXYGEN CONTENT

SITE	MAXIMUM VARIATIONS IN OXYGEN CONTENT OF SUCCESSIVE SAMPLES, VOL % Within Chamber	Between Chambers[a]
Pulmonary artery		
Right and/or left	0.4	
Main		
		+0.5
Right ventricle		
Below pulmonary valve	0.8	
Midventricle	1.0	
		+0.9
Right atrium[b]		
Lower	1.5	
Mid	2.3	
Upper		
		+1.9 (sup)
		−1.5 (inf)
Vena cava		
Superior	0.6	
Inferior	0.8	

[a] +, maximum increase over the more proximal chamber; −, maximum decrease over the more proximal chamber.

[b] Occasionally sampling near coronary sinus will give an abnormally low content out of line with other atrial samples.

CARDIAC OUTPUT MEASURED BY RADIOACTIVE INDICATOR

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SINCE Stewart (7) and Hamilton and associates (2, 4) first became interested in the indicator dilution technic for measuring cardiac output, there have been several attempts to find indicators more easily handled and measured than traditional Evans blue (T-1824). Ideally, such materials must possess the following characteristics: *1*) absence of toxicity; *2*) ready miscibility with blood constituents; *3*) relatively slow removal from the blood stream; and *4*) easy identification and measurement.

Storaasli *et al.* (8), studying blood volumes, found that radioactive iodinated human serum albumin (RISA) with a half-life of 8 days, meets these criteria. Unlike T-1824, there is virtually no loss of RISA from the blood stream 10 minutes after venous injection and only 10% loss after 1 hour. In addition, there is no dependence for stability in the vascular system on the state of plasma proteins. Thus, the only limit to the number of repeat measurements carried out is the total cumulative radioactive dose given.

MacIntyre *et al.* (3, 6), using direct recording apparatus, were the first to use RISA for measuring cardiac output. They cited the following advantages: *1*) blood can be used directly for measurement; *2*) absorption of gamma rays by small thicknesses of material is negligible, so that a direct reading of activity can be made while blood is flowing past the counter; and *3*) accurate timing of dilution curve is easily obtained.

METHOD

INJECTATE

Preparation.—The approximate number of μc/cc of RISA is calculated from the known concentration on the day of manufacture multiplied by the per cent of activity remaining on the day of the test (e.g., per cent of activity, 0.500 on the eighth day; therefore if the initial concentration was 400 μc/cc, 1 cc will contain about 200 μc on the eighth day). The indicator is then so diluted that a carefully measured syringe will contain 10–20 μc in 1–3 cc of injectate. The material is injected as a bolus into a peripheral vein or by catheter into a cardiac chamber or central vessel. When a vein is used, it is best to "flush" the dose im-

mediately with 10–20 cc isotonic saline solution to insure its immediate entry into the main circulation.

Calculation.—Following the injection, the measured syringe (plus any portion of the injection system if the dose was delivered without final flushing) is refilled with RISA to the same point as with the original injectate. This dose is delivered into a 1,000 cc volumetric flask and 1 cc of the diluent is counted in the well counter. This result × 1,000 gives the actual dose of injectate in counts/min.

DERIVATION OF DILUTION CURVE

Fractionation.—By this method, peripheral arterial samples are collected by a #17 or #18 indwelling needle at accurate intervals of 1–2 sec. Any reliable fraction collector may be used.

When the injection is made anywhere proximal to the left atrium, 6–8 sec may elapse before the collection is started, and then done over a 40–60 sec period. Radioactivity is then determined by measuring the radiation of 1 cc pipetted aliquots of each arterial fraction sample in any reliable well counter. A semilogarithmic curve of the radiation may then be plotted, as described for the Evans blue technic, and the cardiac output calculated by the following formulas:

$$F = \frac{I}{\int Cdt}$$

where F = flow rate of blood, cc/sec; I = quantity of injectate, counts/min; C = blood concentration of RISA, counts/min/cc; and t = time, sec.

More simply expressed, the formula is:

$$CO = \frac{I}{C_{av}t}$$

where CO = cardiac output, cc/sec; I = quantity of injectate, counts/min; C_{av} = average blood concentration of RISA during primary circulation, counts/min/cc; t = seconds duration of primary circulation; and $C_{av}t$ = area under curve of primary circulation, counts/min/cc × sec.

The three major disadvantages of the fractionation method are: *1)* low curves of long duration, such as are obtained with low cardiac outputs, major valvular regurgitation, or large central volumes (vascular volume between points of injection and sampling) may sometimes be incompletely recorded during the relatively short period of sampling; *2)* poor curves, which should be repeated, may not be discovered until after the procedure is completed; and

3) tediousness of pipetting and analyzing each individual fraction sample.

Its advantage lies in the high degree of accuracy obtained by the long period of counting carried out on each blood sample (S.E. = ±1%).

Direct recording.—Various forms of direct writing graphs are used to obtain a dilution curve during the procedure. Currently, we are sampling brachial arterial blood by a #17 or #18 Riley needle to which is attached a 3 mm polyethylene tubing running through the split thallium iodide crystal of a Bench type scintillation counter. An Esterline-Angus graphic meter,* in conjunction with a Berkeley rate computer and a Berkeley decimal scaler† gives an immediate direct curve. Others prefer using a standard electrocardiograph or Polyviso Recorder‡ for recording (1).

In any continuous counting system there is a loss of efficiency as compared to direct analysis of the fractionally collected blood samples in the well counter. Correction for this discrepancy is made by comparing the counts/min of the background and of the 10 min final dilution samples as recorded directly on the graphic meter with blood samples analyzed in the well counter. In practice, it is more feasible to derive the dose of injectate "seen" by the counting system by calculating the whole blood volume [injectate/(final dilution − background)] by the well counter and multiplying this by the difference between the 10 min final dilution and the background counts/min recorded by the direct counting system.

$$I' = \frac{I}{FD - B} \times (FD' - B')$$

where I' = amount of injectate as "seen" by continuous counting system; I = injectate radiation as measured in well counter; FD = 10 min final dilution as measured in well counter; B = background radioactivity as measured in well counter; FD' = 10 min final dilution as recorded on graph; and B' = background radioactivity as recorded on graph.

The calculation of cardiac output then is identical with that in the preceding discussion. Figure 1*A* shows a typical dilution curve obtained by this method, and Figure 1*B* the same curve transposed to semilogarithmic paper. Calculation of cardiac output from these data follows:

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* Esterline-Angus Company, Indianapolis, Ind.

† Berkeley Division, Beckman Instruments, Inc., 2200 Wright Ave., Richmond, Calif. Rate computer, Cat. no. 1600; decimal scaler, Cat. no. 2105.

‡ Sanborn Company, Cambridge, Mass.

Sample Measured	Measured by Well Counter, Counts/Min*	Recorded on Esterline-Angus Graph, Counts/Min
Injectate (I)	19,531,000	Unknown (I')
Background blood (B)	1,552/cc	900 (B')
10 min final dilution blood (FD)	5,487/cc	1,900 (FD')

$$I' = \frac{I}{FD - B} \times (FD' - B') = \frac{19{,}531{,}000}{5{,}487 - 1{,}552} \times (1{,}900 - 900) =$$

4,970,000 counts/min

$$CO = \frac{I'}{C_a \bar{t}} = \frac{4{,}970{,}000 \text{ counts/min}}{58{,}280 \text{ counts/min/cc} \times \text{sec}} = 85 \text{ cc/sec} = 5.1 \text{ liters/min}$$

MacIntyre *et al.* found values for cardiac output by this method to agree within 9% of those obtained by the aortic rotameter in dogs (3) and within 8% of those obtained by the Fick method (6) in man. The most frequent causes of error are: *1*) difficulty with injection, i.e., improper measurement, slow entry of indicator from peripheral vein into main circulation, or partial subcutaneous injection; and *2*) error in sampling, especially inconstant flow through the crystal due to partial clotting within needle and/or tubing, or partial occlusion of the needle by arterial wall.

Surface counting.—The necessity of cardiac catheterization for the Fick method and of arterial puncture for dilution methods have limited the widespread clinical use of cardiac output measurement. However, the possibility of obtaining dilution curves by surface counting technics over an appropriate vessel using RISA as an injectate would limit trauma to a single venipuncture.

Storaasli *et al.* (9) have developed such a method for determining hepatic blood flow by surface counting over the liver after injection of RISA into the spleen. They report an error of ±8%, and good correlation with Bradley's bromsulfalein method. Pritchard *et al.* (5) are examining radiocardiograms obtained by counting over the heart after peripheral venous injection of RISA. They find a ±10% correlation of such cardiac output determinations with those obtained by the continuous arterial flow method described above. Crane *et al.* (1) are essaying a method whereby a dilution curve is recorded while the patient holds his finger in the well counter.

Such methods entail calculations similar to those just described, with one difference. In calculating the radioactivity of the final dilution 10 min after injection, the radioactivity in surrounding tissues must be subtracted from the total surface count over the involved vessel. Because there are no large vessels near the liver,

* The background count per minute intrinsic to the counting system is subtracted from the total counts to derive this figure.

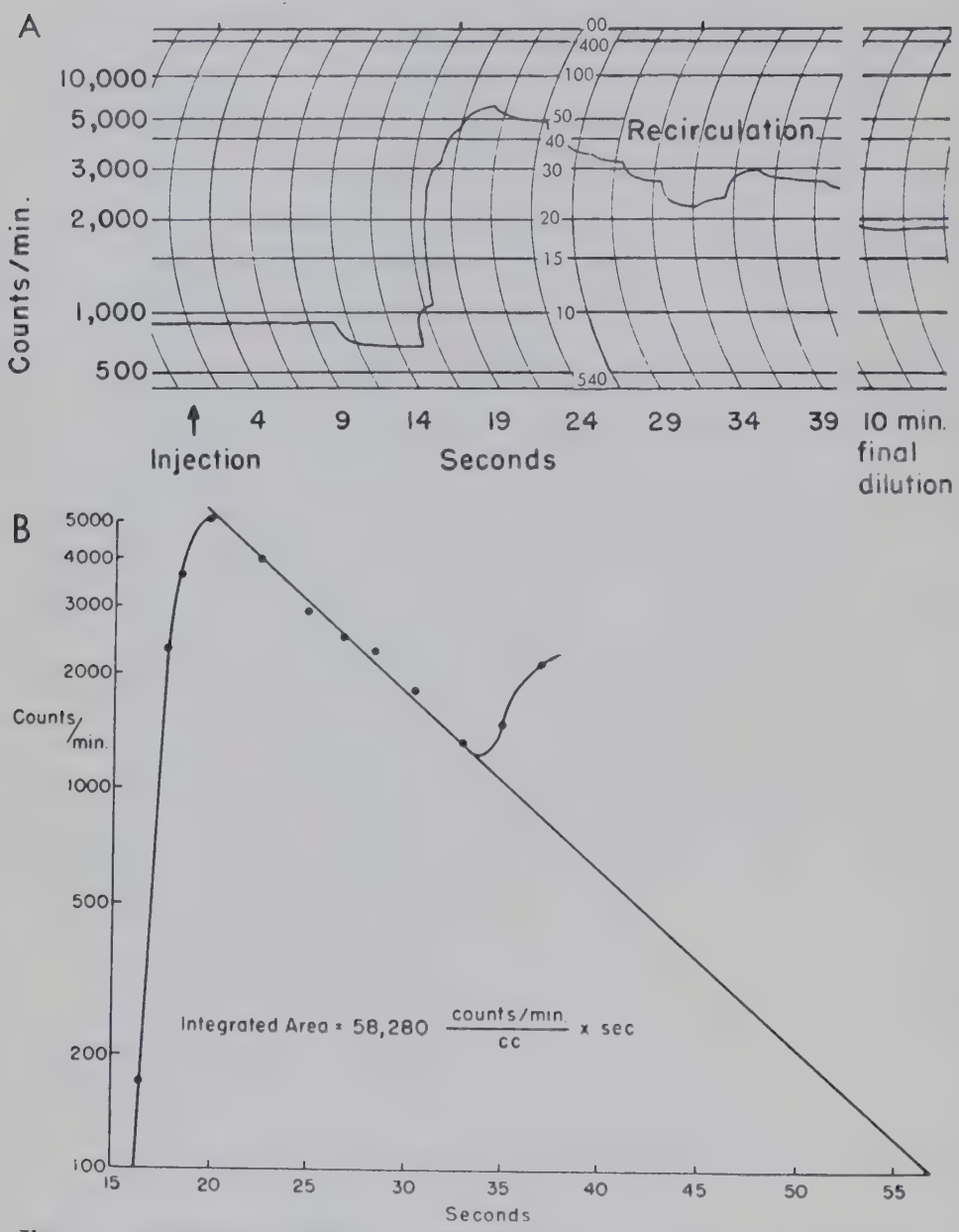

FIG. 1.—Radioactive iodinated (I^{131}) human serum albumin (RISA) dilution curve. *A*, recorded directly on Esterline-Angus graph. *B*, replotted on semilogarithmic paper.

Storaasli *et al.* (9), in calculating hepatic blood flow, measure the radioactivity over the anteroinferior aspect of the shoulder to obtain the tissue radioactivity.

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KORNER-SHILLINGFORD METHOD FOR ESTIMATING REGURGITANT FLOW

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ONE OF THE MORE resistant problems in cardiovascular physiology has been the quantitation of regurgitant flow across an insufficient valve. Korner and Shillingford (3, 4) have recently demonstrated how such information can be estimated from an analysis of the shapes of indicator dilution curves which have been derived by any of the generally employed methods (1).

The shape of an indicator curve is in part a function of the cardiac output (CO) and the volume between injection and sampling sites (CV). A large cardiac output is associated with an earlier appearance time, a lower peak concentration, and a steeper descending limb. A larger central volume results in a later appearance time, a lower peak concentration, and a more gradual downslope. Korner and Shillingford have established that the presence of insufficiency between the injection and sampling sites does not impair the accuracy of the determinations of CO or CV, but that the shape of the curve is so altered that the appearance time is earlier, the peak concentration lower, and the downslope more gradual than for a curve with a comparable CO and CV in the absence of insufficiency. Figure 1 shows the indicator dilution curves obtained by injecting I^{131}-tagged human serum albumin into the left atrium of a patient with pure mitral stenosis (MS) and of one with combined mitral insufficiency and stenosis (MI + MS). The effect of regurgitation upon such curves is a summation of all regurgitant flows between injection and sampling sites.

METHOD

In an effort to translate the qualitative changes described into quantitative measurements, Korner and Shillingford (3) suggested the use of the reciprocal of the downslope (1/s) as a convenient parameter for describing the shape of the curve. This term can be defined as follows:*

$$1/s = \frac{t_2 - t_1}{\log c_1 - \log c_2}$$

where t_1c_1 and t_2c_2 represent the coordinates of two points on the

* Korner and Shillingford used $\log_e C$ in defining 1/s. We have found it more convenient to use the base 10 throughout.

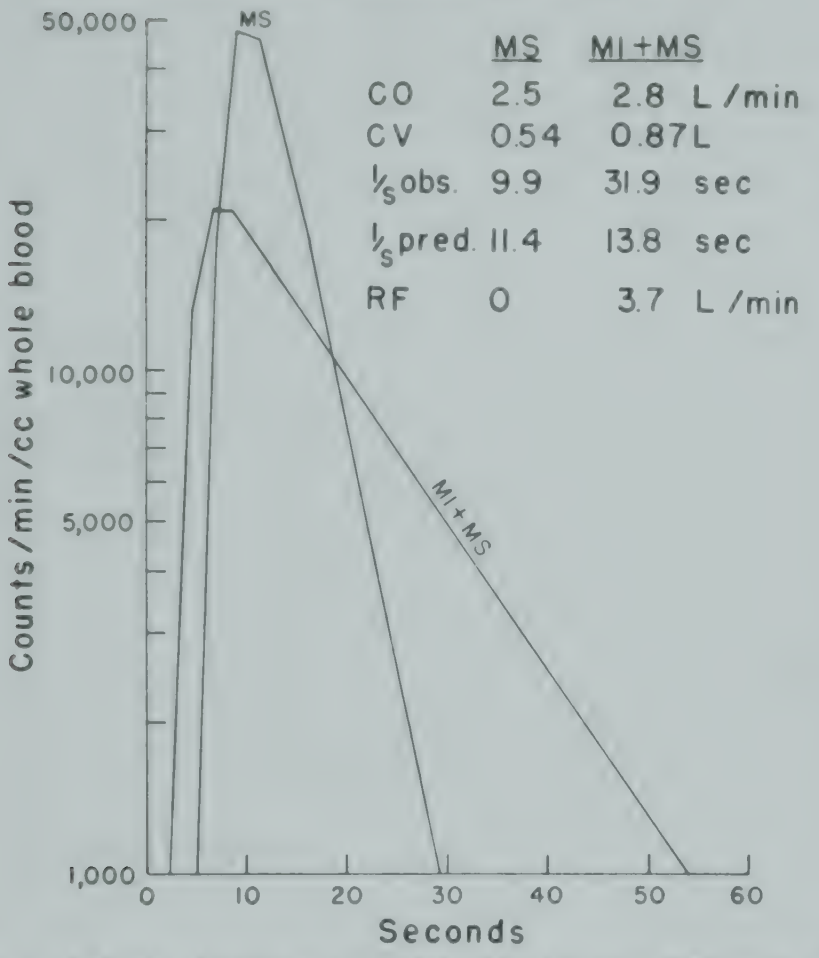

FIG. 1.—Indicator dilution curves obtained by injecting I^{131}-tagged human serum albumin into left atrium in a case of pure mitral stenosis (MS) and combined mitral insufficiency and stenosis (MI + MS).

straight line downslope when the curve is plotted semilogarithmically. If c_1 and c_2 are chosen one logarithmic cycle apart, the denominator reduces to 1, and 1/s becomes simply the number of seconds for the concentration to drop to $^1/_{10}$ of any given value on the straight-line downslope.

If insufficiency or shunts, are absent, they claim, 1/s depends on CO and CV and can be predicted from these parameters by means of multiple regression equations (6) derived from the analysis of curves from patients without valvular insufficiency. Two equations derived by us are:

for a CV less than 1.0 liter (derived from 66 curves)

$$\log 1/s = 1.7298 - 1.2290\ CO + 0.6822 \log CV \qquad (1)$$

for a CV greater than 1.0 liter (derived from 103 curves)

$$\log 1/s = 1.7033 - 1.2369 \log CO + 1.2054 \log CV \qquad (2)$$

CO and CV are expressed in liters per minute and liters, respectively. There is a standard error of ±14% in predicting 1/s by equation (*1*) and of ±28% by equation (*2*).

In the presence of insufficiency, the observed 1/s exceeds the predicted 1/s (see Fig. 1) and the total ventricular output is calculated as suggested by Korner and Shillingford (3, 4):

$$\text{Total ventricular output} = \text{CO} \times \frac{1/\text{s (observed)}}{1/\text{s (predicted)}}$$

and

$$\text{Regurgitant flow} = \text{Total ventricular output} - \text{CO}$$

In patients with mitral stenosis and insufficiency (5), using the left atrium as injection site, it was usually found that when the observed slope was less than 150% of the predicted slope stenosis was predominant; when the observed slope was greater than 250% of the predicted slope, regurgitation was the predominant lesion.

CALCULATION (see Fig. 1)

CO = 2.8 liters/min log = 0.4472
CV = 0.87 liters log = (−0.0605)
1/s (observed) = 31.9 sec

$$\log 1/s = 1.7298 - (1.2290)(0.4472) + (0.6822)(-0.0605) =$$
$$1.1389\ 1/s\ (\text{predicted}) = 13.8\ \text{seconds}$$

$$\text{Total ventricular output} = 31.9/13.8 \times 2.8 = 6.5\ \text{liters/min}$$

$$\text{Regurgitant flow} = 6.5 - 2.8 = 3.7\ \text{liters/min}$$

DISCUSSION

An important objection to this method appears to be the translation of deviation of observed 1/s from predicted 1/s into regurgitant flow in liters/min. Although the validity of this estimation has been demonstrated in models (3, 4), this critical point still remains to be proved in animals and man. Even if this consideration is neglected, a fair error in estimation of the predicted 1/s still exists and hence in the final calculation of regurgitant flow. Furthermore, the accuracy of these regression equations probably diminishes in the low output, high central volume range because of a paucity of such points in the original data from which the regression equations were derived. This defect presumably will be remedied by the continued collection of data with improvement of the regression equation. Korner and Shillingford (4) state that the "variance" of a curve considers the entire shape and is therefore a parameter superior to 1/s. The slight improvement gained, however, seems more than offset by the complicated calculation of variance compared with the simple determination of 1/s.

As has been implied, other factors can alter the shape of an in-

dicator dilution curve in the same direction as insufficiency. The presence of a left-to-right shunt somewhere between injection and sampling sites is one of these factors. Indeed, Korner and Shillingford (3) have suggested that this method could be applied to the measurement of such shunts. The presence of significant shunts can be readily excluded, however, by measuring blood oxygen content in the various chambers.

The presence of a greatly enlarged atrium has been simulated in models and appears to influence the shape of indicator dilution curves. The presence of a stenotic valvular lesion, in the absence of insufficiency, does not alter the shape of these curves other than by way of a small cardiac output (2, 5).

An important factor which needs further investigation is the influence of the type of collecting system used in sampling arterial blood. It is obvious that a very slow flow of blood through a long collecting tube of large volume will in itself distort the shape of an indicator curve. The critical dimensions and flow rates for the collecting system relative to their influence on the shape of the curve have not been established. This factor must be considered when comparing results from different laboratories.*

Finally, since the calculated regurgitation represents the sum of regurgitant flows at all valves between injection and sampling sites, it is impossible to determine by this method the relative contribution of mitral insufficiency and aortic insufficiency when both are present.

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* In our laboratory, arterial samples have been collected in a rotating wheel fraction collector at 1 or 2 sec intervals from a 17 or 16 gage Cournand needle through a 28 cm length of PE 280 polyethylene tubing (volume, 1 cc) at a flow rate between 0.5 and 1.5 cc/sec.

INTRAVASCULAR PRESSURE RECORDING

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THE ACCURATE GRAPHIC registration of the intracardiac and intravascular pulse pressure wave requires a manometer system which will faithfully reproduce the contour as well as the amplitude of the entire pressure wave.

The basic element of all manometer systems is a column of fluid or membrane which is deflected by the pressure variations. To register best the amplitude of a pressure change, the membrane should be a lax or easily deformed one. In addition, if the membrane is to reproduce faithfully the wave's contour, it must respond rapidly to every small pressure change. Unfortunately, the latter requirement necessitates a rigid membrane which resists displacement or distortion. These two membrane properties are obviously incompatible and all manometer systems represent a compromise between them. The use of optic or electric systems which greatly magnify the volume displacement of the membrane has made it possible to employ more rigid membranes displacing little volume through a large given pressure change. The net effect is an apparatus which has a capacity for accurate response to rapid serial pressure variations, i.e., a high natural frequency.

The other factors which modify the frequency response of manometer systems are the length and internal diameter of the catheter and connecting tubes, the frequency response of the galvanometer, and the viscosity of the fluid in the system.

Wiggers (6) has stated that the pulse pressure wave is made up of a series of simple sine waves. From his analysis of these components, he has concluded that the sine waves with a frequency up to the 10th harmonic of the fundamental are of sufficient amplitude to be significant. Nevertheless, Wood (7) has shown that accurate registration of the pulse wave contour can be accomplished by means of any manometer system which has a uniform dynamic response up to 6 to 10 cps. A properly damped system with minimal hysteresis is essential.

The characteristics of a good manometer system may be outlined as follows (3, 7): *1*) high natural frequency; *2*) high stability; *3*) linear calibration; *4*) usability with long leads; *5*) insensitivity to movement; *6*) insensitivity to temperature and humidity; *7*) insensitivity to acceleration; *8*) imperviousness to electrolyte solution; *9*) simplicity of operation; *10*) construction for ease of

sterilization; and *11*) construction for ease of removal of trapped air bubbles.

TYPES OF MANOMETERS

Mechanical Mercurial Column or Water Column

These manometer systems, although accurate for measurement of the mean pressures within the vascular tree or cardiac chambers, are unable to record true systolic or diastolic pressures because of their inertia, and the slow response time registers an extremely overdamped pulse contour.

Optical

By proper design with a rigid small membrane, a manometer system can be constructed that has a low volume displacement and a relatively high natural frequency. The device described by Hamilton (2) has these features. The membranes are difficult for the uninitiated to prepare and to maintain. The recording must be photographic, and relatively short leads are necessary.

Resistance Wire Strain Gages

Resistance wire strain gages (4) are all based on the principle that when a wire is stretched its electric resistance increases in proportion to its change in length. This property is characteristic of a given type of wire. Unbonded strain gages consist of 2 pairs of coiled wires mounted in such a manner that when a stress is applied 1 pair is stretched and the other pair is relaxed. These wires are in a Wheatstone bridge circuit so that a force unbalances the bridge in proportion to the sum of the stretching of one pair and the relaxation of the other pair.

These gages are stable, easy to operate, have minimal drift of the base line, and can be used with long leads. Hysteresis is less than 1% of the pressure change. The strain gage has a linear response throughout its entire range, in fact, to the breaking point of the wire. The resistance wires are so connected that temperature changes are cancelled out while stresses are additive. The main stated shortcoming of this manometer system is its low natural frequency. Although those wishing to achieve high fidelity recordings should utilize a manometer system with as high a natural frequency as possible, there is some question whether significant distortion of the pulse contour occurs until the uniform frequency sensitivity falls below 6–10 cps.

Variable Capacitance Manometer (5)

The principle of this transducer is that the membrane of the manometer system, consisting of a rather stiff metallic diaphragm,

is separated from the electrode by a very small air space and thus functions as one plate of a condenser. Stress on the diaphragm varies the air space between the 2 plates, changing the capacitance, which in turn is measured by means of a radio-frequency circuit.

The main advantage of such a system is its high natural frequency. The manometer when used with a #22 needle has a frequency of 475 cps with less than 1% overshoot. As a result, the transducer can be used in a catheter manometer system employing long small-bore tubing and needles without compromising the integrity of the pulse wave contour. Since the volume displacement is extremely small, little if any blood enters the system to cause clotting. The disadvantage of this system is that the base line stability is poor and the apparatus must be turned on and warmed up for 1 hr before use. This type of apparatus is also sensitive to minor temperature variations.

Variable Inductance Manometer (1)

This manometer system consists of a metal slug which is cemented to a plastic membrane. The iron core lies in the magnetic field of 2 coils. With the application of a stress, the metal core moves in relation to the coils and varies the inductance. Gauer and Gienapp have devised such a transducer which is 1.2 cm long and 2.7 mm in diameter, and can be attached to the end of an 8F Cournand catheter.

The outstanding feature of the transducer is its insensitivity to oscillatory, pendular or rotatory movements. Since its mass is small, it will accurately reproduce the pulse contour even while being subjected to acceleration and deceleration forces as the catheter whips in the pulmonary artery or wedge.

Recording Device

An important feature is that the recording device adds new electronic and mechanical limitations to the system in use. Regardless of the type, whether a direct writing or photographic instrument, its characteristics should be known and its ability to respond studied by the investigator. Limitations of frequency response and inaccuracies in paper speed are of particular significance in checking the performance of the recording device.

PRACTICAL CONSIDERATIONS

1) Regardless of the specific manometer system utilized, it is of course important that the investigator realize the limitations and potentialities of his apparatus for best results. In general, it has been our impression that it is more satisfactory to work with one

or two types of systems in a given laboratory, rather than to intermix various methods. In addition to the characteristics of the type, it has been found that individual units will vary. Such factors as the linearity of response are slightly different from instrument to instrument.

2) Temperature variations may have a profound influence on pressure-sensing devices. Differences may occur depending on whether the instrument is perfused by blood at a temperature somewhat above room level, or by saline solution. Frequently with some devices, handling or holding the pressure-sensitive device may alter its temperature and vary the response.

3) Standardization of the instrument should be carried out each time it is used, and in general it is preferable to standardize both before and after use if one is interested in highly accurate pressure recordings. It is of great aid to make a standardizing device, such as a mercury manometer, an integral part of the apparatus.

4) Small, even minute, air bubbles in the system will ruin the performance of the finest manometer device. Great care should be exerted in filling the system with fluid and making sure that no air is trapped at any point, particularly in blind connecting lengths and at points of the system which cannot be viewed externally. The recent introduction of plastic devices on strain gages has lessened the danger of trapped air in that part of the system. Several suggestions have been made by laboratories for lessening the air hazard. The use of boiled water is probably most satisfactory, since it contains less dissolved air. In some laboratories, pressure systems are flushed thoroughly with carbon dioxide to remove all relatively insoluble nitrogen from the air. Then, when filled with slightly alkaline fluid, the carbon dioxide is readily dissolved and bubbles are less apt to stay in blind pockets. The fluid, if it stands in the apparatus, should contain both antibacterial and anticoagulating agents. Injection of any material contained within the manometer system into the patient must be avoided. Such material may contain particulate matter or irritating chemicals, or may have been the seat of bacterial or viral infection.

5) The selection of a catheter has profound effects on the quality of the pressure recording. Although there is no general agreement, most investigators believe that catheters of a smaller lumen, such as 6–8 F, display less artifact due to motion within the heart than do larger catheters. The reason for this appears to be the smaller mass of blood contained within them. This type of difficulty is, of course, avoided by using a catheter with a pressure-sensitive tip, which, however, introduces other problems of accurate base line

location. Various other methods have been employed to limit the deteriorating effect of catheters on the efficiency of the system. It has not been our experience that the use of inner constrictions, such as catheters within a catheter and similar means, have been particularly helpful. Extreme care is necessary to make sure that the joints between the catheter and the remainder of the system are in excellent apposition and that the connecting tubing, if any, is limited in amount and does not allow leaks or expansion with the small pulsations within the vascular system. It is our feeling that a minimum of connecting lengths, stopcocks, etc., distinctly lessens the likelihood of artifacts or deterioration of the tracing's contour.

6) The system should be kept thoroughly sterile. It has been shown that only a minute amount of contaminated material from the vascular system of one patient may be the means of transmitting the hepatitis virus to another.

7) A monitoring device, such as an oscilloscope, is of great value in ascertaining the position of the catheter tip, in assuring freedom from artifact, and in providing a safety factor of warning in case of arrhythmias.

8) The path of the catheter is important in considering the resultant tracing. If a catheter loop swings freely outside the body, it may induce cyclic changes in the pressure pulse contour. Similarly, inside the body and particularly in the right ventricle, movement of the catheter can contribute serious artifact.

9) An analysis and evaluation of each tracing should be made to eliminate the possibility of confusing artifact. Small flecks of fibrin, contact with a vessel wall, air bubbles, and many other apparently minor factors may result in misleading pressure recordings.

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PULMONARY "CAPILLARY" PRESSURE

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THE SO-CALLED pulmonary "capillary" pressure refers to the pressure measured through a cardiac catheter impacted into a branch of the pulmonary artery in such fashion that there is a free communication between the catheter and the capillary-venous compartment of the lung. The name pulmonary "capillary" pressure was introduced originally by Hellems and co-workers (15) to denote that the pressure so obtained approximated the true pulmonary capillary pressure, the quotation marks emphasizing that it was an approximation only. Lagerlöf and Werkö (16) independently used the term "pulmonary capillary venous pressure." Since then, there has been considerable discussion regarding the name to be applied. The Mayo Clinic group (6) use the term "pulmonary artery wedge pressure," which is probably the most acceptable name in that it implies the method of measurement rather than the site of the recorded pressure.

The pulmonary arteries are end arteries terminating in plexuses of freely communicating capillaries. Precapillary anastomoses between bronchial arteries and pulmonary arteries ordinarily do not occur (20), although recent evidence indicates that they may occur in chronic lung disease such as bronchiectasis (19). The effect of these anastomoses on the validity of the wedge pressure has not been established. Hellems *et al.* (15) first demonstrated that the distal opening of a cardiac catheter, wedged into a smaller division or branch of the pulmonary artery until the vessel was occluded, formed a free manometric system with the pulmonary capillaries and veins of unobstructed regions. Lagerlöf and Werkö (16) subsequently described the phasic pressure variations obtained under these conditions in some detail and demonstrated their similarity to the left auricular pressure contour. They suggested that the pressure so obtained therefore represented a somewhat distorted pressure pulse of the small pulmonary veins or venous end of the pulmonary capillaries.

PROCEDURE

Cardiac catheterization is performed in the usual manner. When the catheter is placed in the right or left pulmonary artery, the patient is instructed to inspire deeply. At the same moment the catheter is rapidly advanced into the peripheral lung field until

the tip is impacted. Visualization of the loop within the heart as the catheter is advanced is essential in that it allows one to judge best when wedging is accomplished, since further advancement at this point only increases the circumference or the bending of the intracardiac loop. The catheter tip must not visibly pulsate, as such motion of the catheter tip synchronously with each cardiac systole indicates that the tip is still free in a smaller branch of the pulmonary artery and is not truly wedged.

Three primary criteria must be met to insure that the wedged catheter is now in communication with the pulmonary capillary-venous network.

1) Blood obtained through the wedged catheter must be fully saturated with oxygen. It is often difficult to obtain blood through the catheter when it is wedged. Syringe aspiration must be gentle. Frequent deep breaths will aid in obtaining blood in some cases. If no blood is obtained, the catheter is withdrawn to the main pulmonary artery and the catheter wedged in another area. This process is repeated until a blood sample can be obtained. Ordinarily, the bright red color of the blood is unmistakable. If uncertainty exists, or in cases in which the pulmonary arterial blood is highly saturated due to left-to-right intracardiac shunt, a sample should be passed through a cuvet oximeter or a sample taken for Van Slyke analysis to insure that blood is fully saturated. Pulmonary capillary blood is always fully saturated, even in cases of cyanotic congenital heart disease and in cases with impaired pulmonary oxygen diffusion, despite peripheral desaturation. The latter is true because local stasis in the wedged area permits sufficiently longer contact of capillary blood with alveolar air to result in full saturation.

2) The contour of the tracing must be consistent with that of an auricular pressure pulse. A distinct, tripeaked phasic contour is usually obtained. At times it is not obtainable, but the contour must be identifiable as auricular and not that of a damped pulmonary arterial pressure pulse.

3) The column of saline in a saline manometer must fall freely to a level approximating the mean pressure obtained through retrograde transmission by one of the many membrane types of manometers, to insure that the tip is not wedged against a vessel wall. Under this circumstance, the saline column placed above the pulmonary "capillary" pressure usually falls less freely and will fail to reach the level recorded by the membrane manometer.

A valid pulmonary "capillary" pressure has been obtained in approximately 75% of cases in which it has been attempted. Often, when the initial few attempts are unsuccessful, further effort may

be abandoned if it is obvious that the wedge pressure in that case is only of academic interest. The percentage of success undoubtedly can be increased proportionally to the amount of time and energy expended in its pursuit. A valid pulmonary "capillary" pressure is hardest to obtain in cases with a high degree of pulmonary vascular resistance, such as in Eisenmenger's syndrome, in idiopathic pulmonary vascular disease, and in other forms of cor pulmonale.

Early in the investigation of pulmonary "capillary" pressures in our laboratory a disturbing number of postcatheterization pulmonary infarctions involving the wedged lobe were noted. The catheters at that time were being sterilized chemically with 1:1,000 aqueous solution of mercurioxycyanide. When this was discontinued and the catheters were autoclaved for sterilization, the difficulty with pulmonary infarction disappeared. Connolly *et al.* (6) found no evidence of pulmonary infarction in a large number of patients in whom wedge pressures were obtained, despite chemical sterilization of their catheters with mercurioxycyanide. It is possible that they flushed their catheters more thoroughly before use and thus eliminated all traces of the chemical. In any case, experience with pulmonary artery wedge pressures is sufficiently great at the present time to state unequivocally that its determination does not add to the risk to the patient above that of routine catheterization.

NATURE OF PULMONARY "CAPILLARY" PRESSURE

Methods are not available for measuring the true pulmonary capillary pressure, either in animals or man. Under normal circumstances of pressure and flow, the gradient of pressure across the pulmonary vasculature is low. In normal man, the pulmonary arterial pressure is, on the average, about 25/9 mm Hg, with a mean of 15. The left atrial pressure is approximately 5–10 mm Hg. Therefore, the pressure across the capillary venous bed of the lungs is at most only a few mm Hg. When these pressures rise as a result of mitral stenosis or left ventricular failure, evidence indicates that the pressure gradient between capillaries and auricle decreases.

Numerous studies, both in animals and in man, have confirmed the validity of the pulmonary artery wedge pressure as an index of pulmonary capillary, pulmonary venous, and left atrial pressure, but there is still some controversy about exactly what the wedge pressure measures. Dow and Gorlin (10) confirmed the agreement between mean pulmonary "capillary" and left atrial pressure in dogs, both at rest and after circulatory stress. Mueller *et al.* (21) noted the same agreement in dogs, and further observed that if

left atrial pressures were sufficiently elevated, their phasic variations were also transmitted to the pulmonary "capillary" pressure contour. Haddy *et al.* (13), on the other hand, were unable to correlate mean pulmonary venous pressures and pulmonary "capillary" pressures in dogs before and after experimental mitral stenosis. It should be noted that in only 1 of the 56 pulmonary "capillary" pressures recorded were they able to obtain an arterialized blood sample through the catheter. Unfortunately, what we consider the single most essential criterion of validity—the presence of fully oxygenated blood—was not met.

Ankeney (1, 2) compared the pulmonary "capillary" and left atrial pressures in dogs before and following venous infusion and pulmonary artery occlusion, and after production of experimental mitral stenosis, mitral insufficiency and pulmonary embolism. He obtained readings that closely followed changes in left atrial pressure, but observed cyclic changes in wedge pressures which at times greatly exceeded left atrial pressures. In his opinion, such phasic variations were artifacts due to improperly impacted catheters which permitted some transmission of pulmonary arterial pressure, and he concluded that the pulmonary "capillary" pressure was a valid index of left atrial pressure only when the curve was flat and devoid of oscillations. It is hard to reconcile Ankeney's conclusions with observations on patients with Eisenmenger's syndrome. In this syndrome, phasic pulmonary capillary tracings with normal pressures have been consistently observed (7), even though pulmonary arterial systolic pressures were at systemic levels. It would appear that appreciable "leakage" of pulmonary artery pressure, with transmission to the wedged pressure, would be incompatible with a normal pulmonary "capillary" pressure under these circumstances. In his experiments, Ankeney did not obtain samples of fully oxygenated blood through the impacted catheters, and the serious question arises whether his observations can be accepted as bearing on the problem of the so-called pulmonary "capillary" pressure. In 2 patients, Werkö *et al.* (24) occluded the lobular pulmonary artery with a balloon while recording the pulmonary wedge pressure distally through a second lumen. They clearly demonstrated tracings of left auricular contour, devoid of pulmonary arterial contamination under these circumstances. Their work has been confirmed in dogs by Mueller *et al.* (21), who found that pressures recorded distally to the obstructed main pulmonary artery reflected phasic left atrial pressure variations which were even less damped than those recorded from the wedged catheter tip.

Connolly *et al.* (6) demonstrated a close correlation both in

magnitude and in contour between left auricular and pulmonary artery wedge pressures when these were recorded simultaneously during mitral valvuloplasty in patients with mitral stenosis. Bjork and associates (3) measured the left atrial pressure by direct right paravertebral puncture simultaneously with the pulmonary "capillary" pressure during the Valsalva maneuver. They noted that in every instance the shape of the pulmonary capillary curve paralleled that of the left atrial pressure curve. Finally, Epps and Adler (11) have compared the pulmonary artery wedge pressure with simultaneous left atrial pressures obtained through closed chest transbronchoscopic needle puncture in unanesthetized patients with mitral stenosis. In all instances, the left atrial and pulmonary "capillary" mean pressures were equal. Furthermore, the pulmonary "capillary" pressure pulse was similar in contour to that of the left atrium in every case. They deliberately selected a wide spectrum of cases with low and high atrial pressures, low and high pulmonary arteriolar resistances, mitral insufficiency, and with sinus rhythm and auricular fibrillation. The recordings are excellent and leave little if any room for doubt that pulmonary artery wedge pressure mirrors left atrial pressure when care is taken to insure a free connection between the catheter and the capillary–venous bed by fulfilling the three criteria mentioned.

The pressure gradient from the pulmonary capillaries to the left atrium may widen with the elevated pulmonary blood flows that occur in left-to-right shunts. Snellen (22), Hellems *et al.* (14), Connolly *et al.* (5), and Lagerlöf and Werkö (16) demonstrated the similarity of mean pulmonary "capillary" pressure and left atrial or pulmonary venous pressures in cases of atrial septal defect. Calazel *et al.* (4), however, were unable to obtain phasic configurations resembling those of the left atrium or even equivalent mean pressures when left atrial pressures were elevated. Weissel *et al.* (23), in most of their cases of atrial septal defect, were able to obtain distinct auricular waves in the wedged pulmonary artery pressure and related the failure of Calazel and associates to the particular recording and damping system which they used. Our own experience indicates that in most cases of atrial septal defect, especially those with only moderate increases in pulmonary flow, the pulmonary "capillary" pressure is the same as the left atrial pressure. With large flows, the "capillary" pressure is often as much as 4 to 10 mm Hg higher than that in the left atrium.

APPLICATIONS AND VALUES

Normal pulmonary "capillary" pressure ranges from 7–12 mm Hg, with an average of 10 mm (14). It rises only 3–4 mm Hg with

moderate exercise (8). Pulmonary "capillary" pressure is equal to, or, if pulmonary arteriolar resistance is increased, is less but not greater than pulmonary arterial diastolic pressure. Ordinarily, distinct "a, c, and v waves" are recognizable, although at times there is apparently sufficient damping by the extensive capillary network or by lax elasticity of the system to result in a rather flat curve. At other times, standing waves, respiratory variations, and motion artifacts of the catheter, which are frequently bothersome in recording all types of pressure during cardiac catheterization, may result in an uninterpretable pulmonary "capillary" pressure curve.

An elevated pulmonary "capillary" pressure either at rest or with exercise generally signifies mitral stenosis or left ventricular failure; more rarely, pulmonary venous compression due to granulomatous involvement, pulmonary vein thromboses, constrictive pericarditis involving the left ventricle, left atrium or pulmonary veins, or myxoma or thrombus of the left atrium obstructing the mitral valve may be responsible.

The ability to estimate left atrial pressure or, in the absence of mitral stenosis, left ventricular diastolic pressure, has been of great value both in physiologic and in clinical studies. The determination of the pulmonary "capillary" pressure in patients with mitral stenosis permits evaluation of the severity of the narrowing and its change after operation, through estimation of the mitral valve area by the Gorlin formula (12). In addition, clinical findings can be correlated with the degree of mitral valve obstruction calculated from physiologic studies, permitting a clinical classification of mitral stenosis into stages of severity (17).

Pulmonary "capillary" pressure, in the absence of mitral valvular obstruction, is an approximation of mean left ventricular diastolic pressure. Elevation of this pressure with exercise is often the first sign of left ventricular failure. When left ventricular failure has progressed to the point where pulmonary "capillary" pressure is elevated at rest, its level when correlated with the stroke work of the ventricle gives a semiquantitative measurement of the degree of failure. Impairment of left ventricular function thus demonstrated may precede clinical symptoms and signs (18).

The pulmonary vascular resistance can be estimated by Poiseuille's formula (8) if the pulmonary arterial and pulmonary "capillary" pressure and cardiac output are known. This resistance is furnished by the arterial end of the capillary bed, the smaller arterioles, capillaries, and perhaps the venules. An elevated pulmonary vascular resistance may be primary or secondary, or at least related to an elevation of the pulmonary "capillary" pressure

(3). Increased pulmonary vascular resistance in the presence of an elevated pulmonary "capillary" pressure is seen in mitral stenosis and, to a lesser degree, in left ventricular failure from any cause. Elevation of pulmonary vascular resistance with normal pulmonary "capillary" pressure is seen in cor pulmonale, idiopathic primary pulmonary vascular disease, Eisenmenger's syndrome, and occasionally in certain cases of congenital heart disease perhaps as a response to long-standing left-to-right shunts.

Contour analysis of the pulmonary "capillary" pressure tracing is of limited value. Extremely high v waves may be seen in mitral insufficiency, but since the volume of regurgitation is only one of several factors determining its height, it is at best only a qualitative measure indicating the presence of regurgitation but not its magnitude. Thus, normal individuals have been observed with large v waves, and v waves of normal size have often been observed in patients with severe mitral insufficiency. When the mitral valve opens, the left auricle and ventricle are in communication, and theoretically the slope of the descending limb of the v wave should be steep in the absence of mitral stenosis and gradual in its presence; the level at which it becomes a plateau should represent the diastolic pressure during diastole in the left ventricle. In general, this slope (fall in mm Hg unit time) is gradual in mitral stenosis and steep in predominant mitral insufficiency, but in our experience too many exceptions have been observed to make this a useful point in differentiating the two lesions in an individual case. Other factors, such as mitral valve flow, the pulmonary blood volume, pressure volume characteristics of the left atrium-pulmonary venous compartment, and the damping effect of the capillary-pulmonary venous network, will influence the slope of the runoff of the v wave in an unpredictable fashion in individual patients. We have ceased to use the level to which the runoff becomes a plateau as a measure of left ventricular diastolic pressure for empiric rather than theoretic reasons.

In summary, to us it seems a mistake to conclude that the pulmonary "capillary" pressure is a true measure, under all circumstances, of left auricular or of pulmonary capillary pressure. It appears, rather to be a measure of the pressure somewhere in that compartment limited on the one hand by the pulmonary capillaries and on the other by the left auricle or, in diastole, the left ventricle, provided that there are no obstructing lesions in between. Since at most the pressure gradient across this circulatory compartment is small, the pulmonary "capillary" pressure can be used as an approximation of the pressure in any and all of these areas, especially in patients with normal or decreased pulmonary

blood flows. From the foregoing, it should be obvious that one cannot split millimeters in interpreting the pressures recorded. On the other hand, a valid pressure can be put to valuable use, as described above, in interpreting events in the lungs and in the left side of the heart.

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TECHNIC OF LEFT HEART CATHETERIZATION

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VARIOUS TECHNICS have been used for the direct measurement of left auricular pressures. Allison and Linden (1) described the transbronchoscopic and transesophageal technics in 1953. In addition to auricular pressure tracings, gradients across the aortic and mitral valves with dye-dilution curves and cardiac output determination, afford information about stenosis, regurgitation and myocardial function.

We have tried the gamut of technics, including the transbronchoscopic and retrograde arterial methods. In general, the tracings were unsatisfactory and complete studies inconstant. A complete study or complete left heart catheterization provides two types of data: *1*) It embraces aortic pressures as reflected by brachial artery tracings, simultaneous left ventricular tracings and subsequent or simultaneous left auricular tracings. *2*) The complete study includes cardiac output determination (Evans blue dye and/or I^{131} dilution curves as injected into the left auricle and recovered at the brachial artery). Right heart catheterization is often done at the same time; however, it is not a requirement for "complete" left heart catheterization. The relatively blind, left lateral decubitus percutaneous technic of Björk *et al.* (2) was not consistently successful and was associated with a high incidence of pneumothorax, cough and hemoptysis. Chesterman's (3) direct ventricular needle puncture seems hazardous.

The percutaneous technic of Kent *et al.* (4) captured our interest and is the basis of our method. Their fluoroscopic control, needle and catheter manipulation have undergone simple modification; the technic was stabilized within the first half dozen trials, and has been quite consistently successful in the first 100 patients.

TECHNIC

The left auricle must be pierced with a needle. That needle should point to the mitral valve. The cardiac silhouette is seen with the fluoroscope from behind. The cardiac apex is then to the left and downward; the right border is made up of the cavae and the auricles. Normally, the left auricle is more above than below the midpoint of the right border. The left auricle may figure more prominently in the right border if the mitral valve is diseased. The auricle in mitral insufficiency may be larger and lower than in stenosis. The mitral orifice generally lies approximately in the mid-

line of the body in a line between the midpoint of the upper half of the right cardiac border and the ventricular apex. This suggests the proper needle direction.

The patient lies face down in the ventral decubitus position on a fluoroscopic table. His left brachial artery has been cannulated. His head is turned to the left and the operator works on the right side of the patient. The surgeon can estimate from the postero-anterior roentgenogram the approximate position on the patient's back representing the midpoint of the upper half of the right cardiac silhouette. The patient's back is widely prepared and draped with sterile sheets, exposing a square area over the heart approximately 30 cm on a side. A pair of sterile scissors is placed transversely across the patient's midline in the position presumed to bisect the right heart border. A sterile towel is then thrown over the field to avoid contamination by the fluoroscopic screen. The operator checks the position of the instrument fluoroscopically to see whether it gives surface orientation of the right border of the cardiac silhouette at its midpoint or just above. If it does not so transect the cardiac silhouette, the fluoroscope is turned off and the operator, wearing sterile gown and gloves, reaches under the sterile towel and makes the appropriate adjustment of the marking instrument. The position is then checked again. Thus the external or surface landmark is determined. When the lights are on and the field exposed, the site for needle insertion is taken from the position of the scissors. The spinous processes define the midline and the longitudinal axis. The scissors define the transverse axis. The needle site in the skin is 5 or 6 cm to the right of the midline in the right upper quadrant of the silhouette. This point is infiltrated with procaine. The 16 gage, thin-wall cardiac needle is then inserted at the point of infiltration. It is directed inward, downward and to the left, i.e., ventrally caudad and leftward. The angle from the right chest wall is approximately 45°; from the transverse plane, approximately 45°; and deviated from the midline about 45° to the right. The needle passes the rib and the body of the vertebra, and slides along the right side of the vertebral body toward the pericardium and auricle which offer characteristic resistance. When this is passed, the needle is in the auricle. The open bevel or face of the needle should point in the general direction of the mitral orifice. The stilet is removed from the needle and blood issues from the needle if there is much auricular hypertension. If the patient is normally oxygenated, this blood should be arterial in character. The operator then picks up the sterile #60 polyethylene catheter and looks for its natural curve. This curve is utilized to search for the mitral orifice. The first tracing records the auricular pressure.

The next step is to probe for the mitral orifice in a series of slow, orderly catheter excursions. The perfusion pump should keep the catheter and the cable to the manometer just overfilled with sterile fluid. The manometer is connected to the writer, which indicates passage of the catheter into the left ventricle.

The peak and mean systolic gradients across the aortic valve are taken from simultaneous brachial artery and left ventricular tracings. We make no attempt to pass the catheter from the left ventricle into the aorta, since this would require a less satisfactory catheter. The brachial tracing provides an acceptable representation of aortic pressures.

The aortic valve gradient having been determined, the manometer sensitivity is appropriately altered for diastolic gradients, and the catheter is withdrawn from the ventricle, across the mitral valve into the left auricle. The catheter is then left in the auricle for the injection of I^{131} or dye. These dilution curves not only help determine cardiac output but mitral and aortic regurgitation. If simultaneous ventricular and auricular tracings are essential, a separate #18 needle can be inserted into the auricle.

This simple technic, properly used, provides consistently successful left auricular and left ventricular tracings. When combined right and left heart catheterization is carried out, the right heart catheter is placed first and rests in the pulmonary artery as the patient is turned face down for the left heart manipulation.

It is not uncommon for the patient to have hemoptysis following this procedure. The patient will not be alarmed about this if forewarned. Vigorous coughing may render left heart catheterization unsafe. While we have had no fatal accidents, three alarming "vagal reactions" with bradycardia indicate that electrocardiographic monitoring is essential and that atropine should be ready for intravenous injection. The cardiac pacemaker and cardiac resuscitation equipment are in the room. The significance of ventricular premature beats as a contraindication to this procedure is presumably the same as in right heart catheterization.

Direct aortic, left ventricular and right auricular punctures have occurred without incident. If the first thrust of the needle does not locate the left auricle but is well tolerated, second or even third attempts seem proper. Second attempts should be necessary in fewer than a third of the patients. Complete studies should be possible in better than 80% of cases.

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CALCULATION OF VASCULAR RESISTANCE

JAMES V. WARREN *and* RICHARD GORLIN

RESISTANCE MAY BE defined as the impedance to blood flow through a given portion of the circulation; this may be a total circuit, such as pulmonary or systemic, a region, such as the forearm, or an organ, such as the kidney. Resistance is usually described in terms of pressure loss per unit of blood flow delivered.

Although there is considerable debate regarding the exact anatomic location and physiologic basis of vascular resistance, the concept appears to have practical value. It has been recognized in the field of nonbiologic hydraulics that resistance of multiple pipe systems can be calculated by application of the principles of Ohm's law. Circulatory resistances, by analogy, are calculated similarly. Thus, the simplest formula for calculating resistance is:

$$\text{Resistance factor} = \frac{\text{Arterial mean pressure gradient (mm Hg)}}{\text{Blood flow (cc/sec)}}$$

In recent years, however, many workers in this field have made use of Aperia's formula, which treats the above data so as to give the result in absolute values.

$$R = \frac{P_1 - P_2 \times 1{,}332}{CO} = \text{dyne-sec/cm}^5$$

where R = resistance; $P_1 - P_2$ = pressure loss across resistance, mm Hg; and CO = blood flow through resistance, cc/sec.

Accordingly, the pressure drop across the resistance to be studied must be determined. Usually, both entrance and exit pressures are measured. In practice, however, the exit pressure for the systemic circuit is so little related to the entering pressure that it is often disregarded. However, in the pulmonary vascular bed this is far from true, and the actual pressure gradient should therefore be measured. The calculated values for resistance are based on measurements of arterial blood pressure and blood flow; since these are subject to considerable error, the resultant value may be erroneous by similar magnitude. Minor variations in resistance are therefore usually of no significance. Observed changes in resistance should always be considered in the light of changes in perfusing (arterial) pressure, or, in the pulmonary circuit, alterations in pulmonary venous pressure as well. Changes in the apparent resistance may result merely from the distending effect of the required inflow or exit pressure, and, conversely, a change in the

peripheral bed may be masked by alteration in the distending forces. A final consideration in interpreting values is the effect of body size; the validity of correcting for surface area, as in the case of the cardiac output, is debated.

Just exactly what is measured by "resistance" remains problematic. According to Poiseuille's law, resistance varies directly with vessel length and blood viscosity, and inversely with cross section area. Other factors being equal and unchanged in the same patient, changes in resistance are presumed to reflect changes in cross-sectional vascular area. A decrease in resistance could be due to increased vascular distention by higher entering pressure or to true vasodilatation.

CALCULATION OF CARDIAC WORK

R. GORLIN *and* S. G. GORLIN

CARDIAC WORK HAS two components: work done to deliver blood at a given pressure and work done to impart velocity to the blood. The formula for work is:

$$W = P \times dV + \frac{mv^2}{2g}$$

where P = mean ventricular systolic pressure; dV = volume of blood ejected; m = weight of blood ejected; v = mean velocity of blood during ejection; and g = gravity acceleration. Ordinarily, over 95% of the work of the ventricles is done in creating pressure, and only the remaining component is expended on velocity. For practical purposes, therefore, only "pressure work" is calculated.

The conventional formulas for work in gm M/beat delivered to the pulmonary (W_p) and systemic (W_s) circulations respectively are:

$$W_p = \frac{RV_{sm} \times SO \times 1.36}{1{,}000}$$

$$W_s = \frac{LV_{sm} \times SO \times 1.36}{1{,}000}$$

where RV_{sm} = right ventricular systolic mean pressure, mm Hg; LV_{sm} = left ventricular systolic mean pressure, mm Hg; SO = stroke output, cc/beat; and 1.36 = mercury conversion factor (mm Hg to cm H_2O).

One must carefully distinguish between work delivered to a given circulation (pulmonary or systemic) and work generated by a given cardiac chamber. Ordinarily, there is little difference, for example, between "left ventricular" work and "aortic" work. This was implied by Landowne and Katz (1) in their discussion of cardiac work. They state, "These formulas ignore the small amount of pressure and velocity energy the blood contains when it reaches the ventricles. . ."

In calculating precisely the work performed by *each* ventricle in the presence of cardiac disease, however, it may be important to consider the diastolic energy contribution to each ventricle from its mate. The ventricles function as a multiple-stage pump system; the contribution in work by each ventricle during systole actually begins with the pressure level immediately preceding systole, i.e., end diastolic pressure. Hence, pressure delivered by the left

ventricle equals $LVsm - LVd$. LVd was created by the right ventricle. Similarly, pressure for the right ventricle equals $RVsm - RVd$. In the presence of cardiac failure or pericardial compression, the pressure contribution by the other ventricle may be considerable (as high as 50%). Failure to take this factor into consideration may lead to wrong conclusions about ventricular function. High filling pressure and associated chamber dilation may result in increased energy consumption by the ventricle during contraction and frequently in a lowered efficiency. Such factors, however, bear no relation to the calculation of the useful work actually performed by the ventricle in question.

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CALCULATION OF ORIFICE AREAS WITHIN THE CARDIOVASCULAR SYSTEM

RICHARD GORLIN

A STANDARD HYDRAULIC FORMULA for orifices has been applied to certain fixed orifices within the circulatory system (5). The general formula relates the drop in pressure across the orifice to the rate of flow through it:

$$A = \frac{F}{C_1 C_2 \sqrt{2gh}} = \frac{F}{C \times 44.5 \sqrt{P_1 - P_2}}$$

where A = orifice area, cm^2; F = flow rate, cc/sec; C_1 = velocity coefficient; C_2 = orifice contraction coefficient; $C = C_1C_2 \times 1.17$;* $44.5 = \sqrt{2g} = \sqrt{1{,}960}$ = acceleration factor; and $h = P_1 - P_2$ = orifice pressure gradient, mm Hg.

SPECIFIC APPLICATIONS

In each case the rate of flow must be calculated for that period when the particular valve in question is open: diastole for atrioventricular and systole for semilunar valves. When possible, an empiric constant must be derived for each valve from a comparison of anatomically measured and physiologically calculated areas. When not available theoretic constants are used.

Mitral Stenosis (5)

$$MVA = \frac{CO/DFP}{0.7 \times 44.5 \sqrt{\text{“}PC_m\text{”} - 5}} = \frac{CO/DFP}{0.7 \times 44.5 \sqrt{LA_m - LV_{md}}}$$

where MVA = mitral valve area, cm^2; CO = cardiac output, cc/min; DFP = diastolic filling period, sec/min = diastolic filling period, sec/min × heart rate; 0.7 = empiric constant, C; 44.5 = gravity acceleration factor; LA_m = left atrial mean pressure; “PC_m” = pulmonary capillary mean pressure, mm Hg (3, 4); LV_{md} = left ventricular diastolic mean pressure, mm Hg; 5 = assumed left ventricular diastolic pressure, mm Hg.

The sources of error are:

1) Flow rate. When there is sizable associated regurgitation, the usual methods of blood flow measurement will estimate only the *effective* flow, not the total flow (which includes that lost via regurgitation) through the valve. The method of Korner and Shillingford (6) for estimating the volume of regurgitation offers

* The square root of the factor, to change mm Hg to cm H_2O.

promise of arriving at a more accurate total flow rate through the mitral valve.

2) *Left atrial pressure.* This may be recorded by left atrial puncture (1, 2), and is usually accurate.

3) *Pulmonary capillary pressure.* Multiple recordings must be made until a reproducible value with atrial-like pressure pulse is obtained, if direct left atrial pressure is not used in the formula.

4) *Assumed left ventricular diastolic pressure.* Ordinarily, this is not a source of error, unless left ventricular failure coexists. This error is eliminated if left ventricular diastolic pressure is recorded directly (2). It must be borne in mind that when the valve gradient is small, the chance of error in the area calculation is larger.

Results.—A survey was conducted among 28 investigators in the cardiovascular field. Approximately half used the formula and found it of definite help. The others either did not use it or found it of no help.

Among 10 workers, including our large series, 7 reported a 75% or better correlation of calculated and measured valve areas. The other 3 found the calculated areas "unreliable." These investigators commented on the same sources of error as mentioned above, and most, including the author, believed patients with tight stenosis could usually be selected clinically. The two main values of the formula, as expressed by those who used it, were that it gave a better understanding of stenotic valve disease, and that it was an aid in the occasional patient in whom clinical opinion was in doubt.

Tricuspid Stenosis

A similar calculation may be made as for mitral stenosis, utilizing right atrial diastolic mean pressure minus right ventricular mean diastolic pressure as the gradient. The regurgitation factor remains the major source of error, but may be obviated by use of the Korner–Shillingford technic (6).

Pulmonic Stenosis

$$PVA = \frac{CO/SEP}{1.0 \times 44.5\sqrt{RV_{sm} - PA_{sm}}}$$

where PVA = pulmonic valve area, cm^2; CO = cardiac output, cc/min; SEP = systolic ejection period, sec/min = systolic ejection period, sec/beat × heart rate; 1.0 = empiric constant; RV_{sm} = right ventricular systolic mean pressure, mm Hg; and PA_{sm} = pulmonary arterial systolic mean pressure, mm Hg.

The major source of error is in the *flow rate.* Associated regurgitation will introduce an error similar to that in mitral stenosis.

Aortic Stenosis

A similar calculations may be made, utilizing left ventricular systolic mean pressure and arterial systolic mean pressure. An error is introduced if there is associated regurgitation, but this may be decreased by calculating the volume of regurgitation by means of the Korner–Shillingford method. Unless great care is taken, the method of measuring left ventricular systolic mean pressure via transatrial catheterization of the left ventricle may introduce errors due to damping (2).

A constant of 1.0, theoretically derived, is being used at present (7).

Regurgitant valve areas may be calculated in *mitral insufficiency* and *aortic insufficiency*, using regurgitant flow as calculated from the Korner–Shillingford method. For *mitral insufficiency*, the formula is:

$$MRA = \frac{RO/SEP}{31\sqrt{LV_{sm} - LA_m}}$$

and for *aortic insufficiency:*

$$ARA = \frac{RO/DFP}{44.5\sqrt{A_{dm} - LV_{md}}}$$

where MRA = mitral regurgitant area, cm^2; RO = regurgitant output, cc/min; LV_{sm} = left ventricular systolic mean pressure, mm Hg; LA_m = left atrial mean pressure, mm Hg; ARA = aortic regurgitant area, cm^2; A_{dm} = aortic diastolic mean pressure, mm Hg; and LV_{md} = left ventricular diastolic mean pressure, mm Hg.

Calculation of area may be made for *small atrial septal defect:*

1) Gradient: $LA_m - RA_m$
2) Flow rate: throughout cardiac cycle, therefore $CO/60$

for *ventricular septal defect:*

Method I: *1*) Gradient: $LV_m - RV_m$
2) Flow rate: $CO/60$
Method II: Phasic calculation (5)

and for *patent ductus arteriosus:*

1) Gradient: $A_m - PA_m$
2) Flow rate: $CO/60$

where LA_m = left atrial mean pressure, mm Hg; RA_m = right atrial mean pressure, mm Hg; LV_m = left ventricular mean pressure, mm Hg; RV_m = right ventricular mean pressure, mm Hg; A_m = arterial mean pressure, mm Hg; PA_m = pulmonary arterial mean pressure, mm Hg; CO = cardiac output, cc/min; and 60 = sec/min.

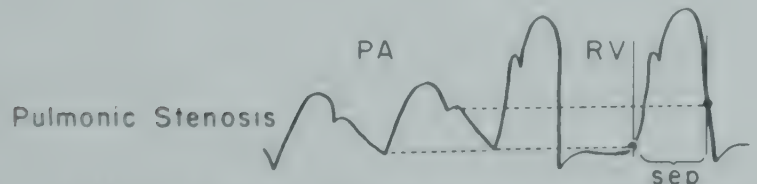
SPECIFIC METHODS

1) For cardiac output, any standard technic, such as Fick's or an indicator dilution method, is adequate. The latter is preferable if regurgitation is to be evaluated quantitatively.

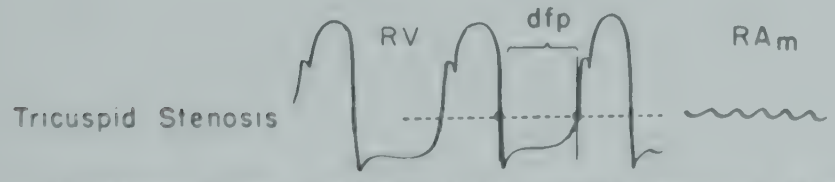
2) For pressures, phasic and mean pressure recordings should be taken simultaneously or in immediate sequence across the orifice being studied, whenever possible, and as close as feasible to the measurement of cardic output.

3) For flow periods, the systolic ejection period (*SEP*) is measured from the initial upstroke of the arterial pulse to the dicrotic

Diastolic filling period (dfp) of left ventricle from brachial arterial pressure tracing



Systolic ejection period (sep) of right ventricle from right ventricular pressure tracing.



Diastolic filling period (dfp) of right ventricle from right ventricular pressure tracing.

FIG. 1.—Measurement of valvular flow periods on pressure pulses.

notch. The diastolic filling period (*DFP*) is measured from the dicrotic notch to the initial upstroke of the arterial pulse. The systemic arterial pulse is used for calculating flow periods for left ventricular valves, and the pulmonary arterial or right ventricular pulse for right ventricular valves (Fig. 1).

A *typical calculation* for mitral stenosis follows. The data are: Cardiac output, 5,300 cc/min; diastolic filling period, 0.31 beat and 34 sec./min; pulse rate, 108; pulmonary "capillary" pressure,

39 mm Hg; left ventricular mean diastolic pressure (assumed), 5 mm Hg.

Mitral valvular flow (MVF):

$$\frac{\text{Cardiac output}}{\text{Diastolic filling period/min}} = \frac{5{,}300 \text{ cc/min}}{34 \text{ sec/min}} = 156 \text{ cc/sec}$$

Mitral valvular pressure gradient:

$$\text{"PC"} - 5 = 34 \text{ mm Hg}$$

Mitral valve area (MVA):

$$\frac{156}{31\sqrt{34}} = 0.9 \text{ cm}^2$$

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KINETOCARDIOGRAPHY*

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PALPATION OF PRECORDIAL movements has been recognized by clinicians for years as a basic technic in the diagnosis of cardiovascular diseases. A heaving precordium is considered by many physicians as one of the more reliable signs of right ventricular hypertrophy, superior even to the electrocardiogram or fluoroscopic examination. A thrusting and forceful apex impulse is of equal value in the diagnosis of left ventricular hypertrophy. The present technic of recording low frequency precordial movements was undertaken in an attempt to establish a more precise definition of these motions. In addition, it appeared possible that more exact characterization of the precordial movements from various points over the precordium would yield information not previously available for evaluating the mechanisms of ventricular contraction and relaxation. The present technic offers a means by which low frequency precordial movements can be recorded easily from any point over the chest wall. The tracings are reproducible from the same points in a given patient from time to time, and similar tracings are noted from comparable points in different normal subjects (3, 4).

TECHNICS

The apparatus and arrangement used in obtaining the kinetocardiogram are illustrated in Figure 1. The sensing unit currently employed is a small metal bellows commercially available from many sources.† The bellows is composed of two 2 inch flanges, each with 2 circumferential corrugations. The size and exact type of bellows does not necessarily have to be like the one described; many other types of bellows have been tested and found to be suitable. The end piece, which is applied to the chest wall, is approximately 7 mm in diameter and is mounted on a 2 inch arm. The open end of the bellows is connected by means of rubber tubing to a piezoelectric transducer. The length of the tubing must be kept short (below 2 ft) so that the resonant frequency of the tubing will be as high as possible, and out of range of the frequencies encountered in the precordial movements. This prevents the intro-

* Aided by a grant H-1914 from the U. S. Public Health, National Heart Institute and Life Insurance Research Grant.

† The bellows we use now was obtained from the Fluez Instrument Division of the Bendix Aviation Corp., Baltimore, Md.

duction of phase shift as the result of tubing factors, as well as changes in linearity. The Cambridge piezoelectric transducer was found to be the most sensitive for this technic and can be used with any standard recording apparatus. The bellows is mounted on a crossbar over the patient so that it can be applied perpendicularly to the chest wall. With this assembly, tracings can be obtained from any desired point. Ideally, the bellows should be applied against the chest wall with standard pressure. However, this is not

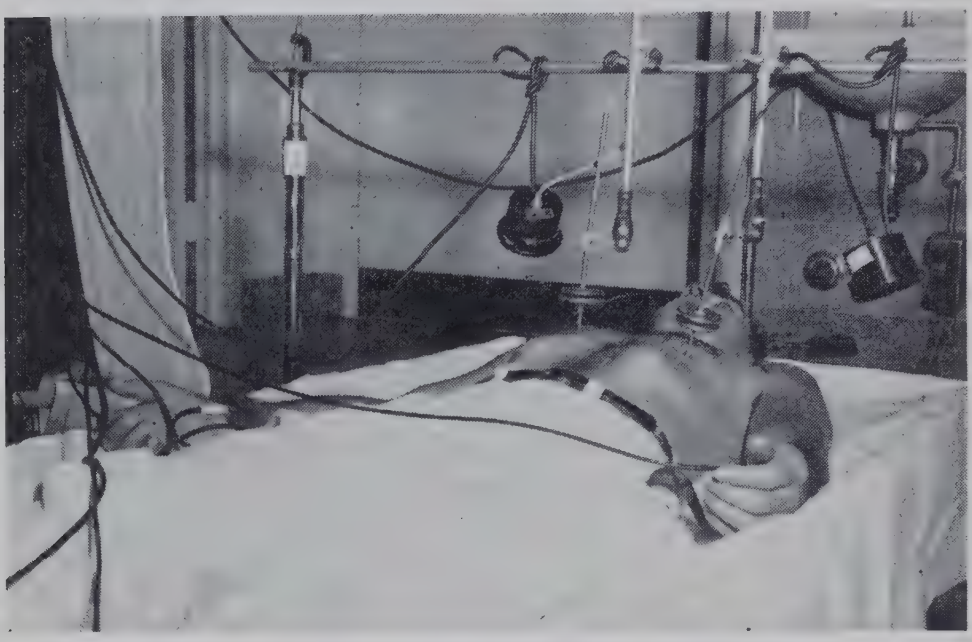

FIG. 1.—Photograph of the apparatus and arrangement used in obtaining the kinetocardiogram. Note that the bellows is suspended from a metal crossbar so that it may be placed perpendicular to any desirable point on the chest. The bellows is connected by means of rubber tubing to the piezoelectric transducer, the output of which is recorded directly on a Poly-Viso through the preamplifier.

absolutely necessary so long as the bellows remains in firm contact. Some variations in amplitude of the tracings occur, depending on the pressure of the pickup device against the chest wall, but there are no changes in configuration. The end piece is usually applied between, rather than on, the ribs. Again, this is not necessary, although the tracings obtained from the interspaces are much sharper than, but not different in contour from, those obtained from adjacent ribs. Obtaining tracings from a reference point outside the body by mounting the bellows on a crossbar has been found to be advantageous over other types of pickups which rest directly on the chest wall.

The bellows type of pickup has been tested against a mechanical

oscillator constructed so as to produce a sine wave of varying frequencies and amplitude. The bellows arrangement is linear in the range of displacement usually encountered in chest wall motion (0.001–0.006 inch), and the amplitude response does not change with varying frequencies (0.6–10 cycles sec). There is no phase shift in this range of frequency when compared to a pure displacement tracing obtained with a photoelectric cell. The bellows used in this laboratory has a resonant frequency response of 75 cycles sec, which is well above the frequency range of low frequency precordial movements and is probably the main factor responsible for the absence of phase shift in the low frequency movements.

Any type of displacement sensing unit can be used. However, it is necessary for the probe to have sufficient travel to encompass the range of motion of the precordial impulses, and, in addition, allow enough travel so that placement on the precordium can be made as easily as possible. Other types of displacement sensing units give essentially the same types of tracings with only minor variations. An Arbeit DVA ballistocardiographic apparatus,* with a $\frac{1}{2}$ inch travel sensing unit, has been used. With this instrument, displacement, velocity and acceleration curves are theoretically possible. However, preliminary observations on velocity and acceleration tracings have shown these to be of such complex nature that interpretation at the present time is impractical. In addition, acceleration tracings tend to magnify high frequency events and will introduce heart sound phenomena. Displacement tracings with this type of linear technic have only minimal representation of heart sounds because of the large displacement encountered in the low frequency movements. It is possible that after sufficient information is obtained about the mechanisms of the low frequency displacement records, velocity and acceleration tracings may be of some value. The presence of heart sounds and other high frequency phenomena is an objection which theoretically limits their over-all usefulness.

Tracings are recorded during the period of held respiration at the end of normal expiration. This is necessary because respiratory excursions of the chest wall are of large magnitude and interfere appreciably with the recording of low frequency displacement phenomena. This is somewhat of a disadvantage, since severely dyspneic patients are unable to hold their breath long enough for adequate registration of the precordial movements. Attempts to overcome this difficulty by using other types of sensing units are

* Industrial Development Laboratories, Inc., 17 Pollock Ave., Jersey City, N. J.

now being tested, but an adequate technic has not yet been developed.

Records are usually obtained from points over the precordium corresponding to the V leads of the electrocardiogram (designated as K_1, K_2, or KV_1, KV_2, etc.); these points are directly over the major cardiac mass, and offer the convenience of utilizing well-recognized points over the precordium. Usually, K_6 yields only

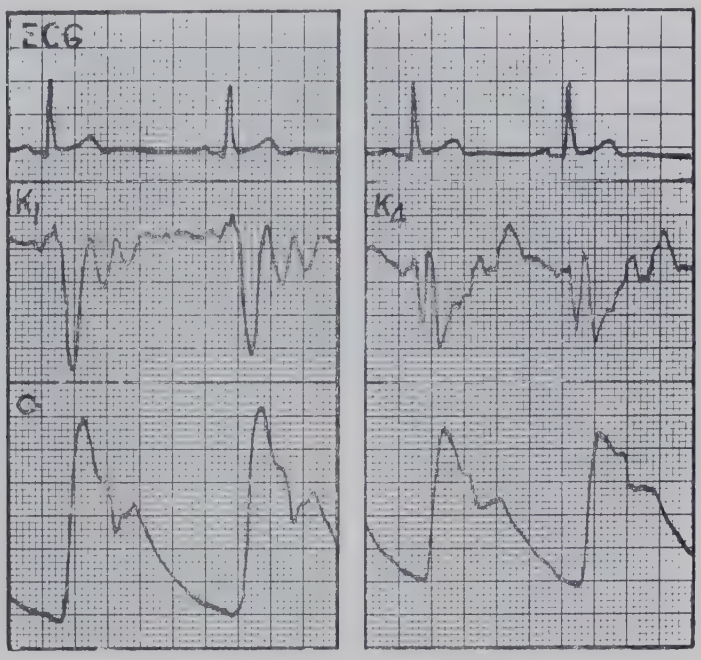

FIG. 2.—Representative tracing obtained from a normal young subject. K_1 tracing corresponds to the V_1 position in the precordial electrocardiographic lead, and K_4 to the V_4 position. K_2 and K_3 tracings are not presented; however, their configuration is of transitional quality between the V_1 and V_4 tracings. The movement corresponding to the palpable apex impulse is indicated by an arrow in K_4. Both K_1 and K_4 positions show a marked retraction during the ejection phase of systole.

minimal information except in very enlarged hearts. In addition, K_3R (same as the V_3 position, except on the right side of the chest) is often of value, especially in patients with right ventricular hypertrophy. Records routinely taken in this laboratory from points over the aortic, pulmonic and epigastric areas suggest that they may be of definite value. However, in the routine situations, K_1–K_5 and K_3R appear to offer the most information. In individual patients, it is occasionally necessary to map the precordium in order to evaluate fully the distribution of the various movements. Figure 2 is a representative tracing (K_1 and K_4 position) obtained from a young, normal subject.

CHARACTERISTICS OF PRECORDIAL MOVEMENTS

Frequency Range

Fourier analysis of representative tracings, obtained with the bellows type of sensing unit, have been made by Drs. Frank Noble and Bert R. Boone at the National Institutes of Health, Bethesda, Md. These tracings were found to have their major frequency components below 15 cycles/sec. There are some higher frequency components which extend as high as 25 cycles/sec, but they are much less significant. Although it is likely that the bellows type of pickup will respond equally well to higher frequencies, at least up to 75 cycles/sec, they are minimized in linear tracings such as these because of the much greater amplitude of the lower frequency movements in relation to the higher frequency components. Thus, the frequency range of the KCG movements (at usual recording sensitivity) is below the range generally attributed to heart sounds (heart sounds are only rarely detectable in kinetocardiograms).

Amplitude of Precordial Movements

The amplitude of the various movements may be estimated indirectly by comparing the displacement to that produced by a mechanical oscillator of a known amplitude (keeping the recording apparatus at the same sensitivity). This is possible because the bellows sensing unit has a linear response. Thus, the amplitudes of major precordial movements in most instances are between 0.001 and 0.008 inch displacement; however, the apex thrust in patients with left ventricular hypertrophy is often of greater magnitude and occasionally the bellows will not be able to follow the movement in its entirety. This occurs only rarely and has not been found a limiting factor in the bellows technic. As the motions recorded are quite small and the amplitude is probably affected by many physiologic variables, reproduction of the amplitude of a given precordial movement in a single patient from time to time is not as consistent as might be desired. So far, only studies of configurational changes and changes in time relationships of the movements have been made (1, 6, 7, 9). The structure of the chest wall undoubtedly influences the amplitude of the precordial movements. As this is a difficult factor to evaluate clinically, the absolute amplitude of the various movements in disease has not been considered in detail. It is important to point out that the chest wall differences alter the configuration of the precordial movements only slightly and in most instances are not a problem.

Effect of Chest Wall

Some of the characteristics of the chest wall have been determined by cadaver studies. By tapping the chest wall with the pickup in place, it was found that the chest wall vibrates at approximately 75 vibrations/sec (4). The resonant frequency of the chest wall may possibly be higher than 75 cycles/sec, as the bellows resonant frequency in this laboratory is also 75 cycles/sec (4). Thus, the resonant range of the sensing unit and of the chest wall are both well outside the frequency range of the precordial movements. Additional studies on cadavers have shown that an outward tap on the chest wall, with the hand inserted into the thoracic cage from an incision in the abdomen, is reflected by an outward movement of the chest wall returning to the resting level without any overshoot (4). The outward movement was greatest in magnitude if the tap was made directly beneath the pickup; however, taps at adjacent areas were still registered, although of smaller amplitude.

Relation to Apex Cardiograms

Kinetocardiograms have been compared to tracings obtained by the use of a funnel applied directly to the chest wall and connected to a piezoelectric transducer (apex cardiogram) (4, 8). Although frequently the tracings obtained with a funnel are similar to the kinetocardiographic tracings, a pickup device which rests directly upon the chest, while tending to neutralize the large swings of the respiratory movements and thus allow tracings to be obtained during respiration, should not be used for several important reasons:

1) The configuration of the tracings largely depends on the size of the diaphragm resting upon the chest wall. Thus, with diaphragms of different diameters, different tracings are obtained which interfere with the standardization of the technic unless everyone chooses a pickup with the same outside diameter.

2) As the output of the pickup device resting primarily on the chest wall depends on the relative motion between the rim and the enclosed surface of the chest wall, only relative interspace motion is recorded. Thus, variations in the records may be expected, depending on the width of the interspace; consequently point chest wall motion (kinetocardiogram) cannot be accurately registered.

3) Large movements of the entire precordium may be considerably minimized by any technic in which the sensing unit rests directly on the chest wall. This is considered important, instances having been encountered in which there is a heave of the entire precordium that is not localized to just one point (6).

4) Previous studies have failed to reveal a consistent pattern for apex cardiograms (10) in contrast to the reproducible findings noted in the kinetocardiographic tracings (3, 4).

Mechanisms of Precordial Impulses

The mechanisms responsible for the various movements registered on the kinetocardiogram are obscure at the present time,

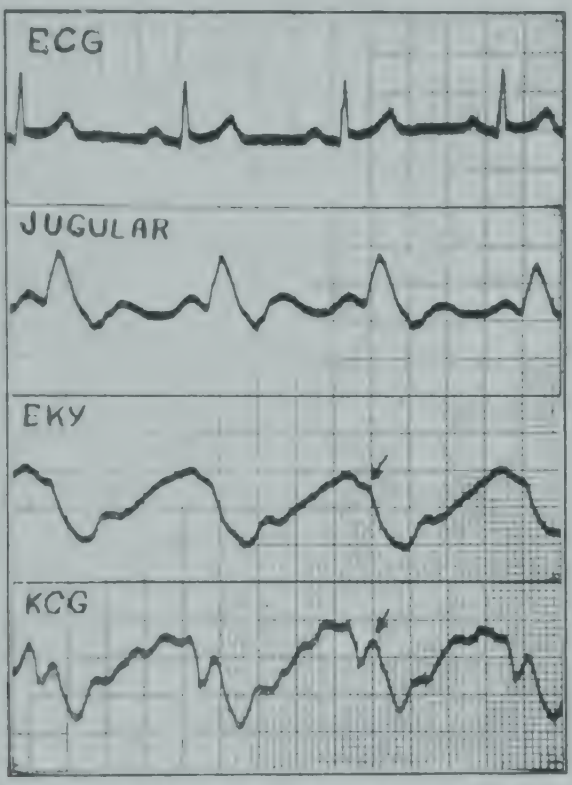

FIG. 3.—Electrokymographic tracing taken from the heart border just beneath the pickup end piece of the kinetocardiographic arm. By this arrangement, a simultaneous heart border motion and kinetocardiogram can be obtained from the same point. The apex thrust is indicated by an arrow. The marked similarity of the tracings suggest that kinetocardiographic movements in the apical region of the heart reflect motions of the underlying myocardium.

and only certain generalizations can be made. Tracings from the region of the cardiac apex probably represent major cardiac movements, as simultaneous kinetocardiograms and electrokymograms from the same point resemble each other both in contour and in time, if a lag of approximately 0.02 sec is considered in the electrokymographic apparatus (Fig. 3) (5). The apex thrust is registered in both tracings, although somewhat slurred over in the electrokymogram. Intrathoracic volume changes or shifts in blood also probably influence the tracings, especially during early ejection.

as there is a symmetric retraction of the entire precordium as well as of the posterior aspects of the chest at this time. In addition, some of the movements, probably during the periods from the onset of the QRS complex to the beginning of ejection and isometric relaxation phase, may be in part influenced by forces associated with myocardial contraction and relaxation. This is pos-

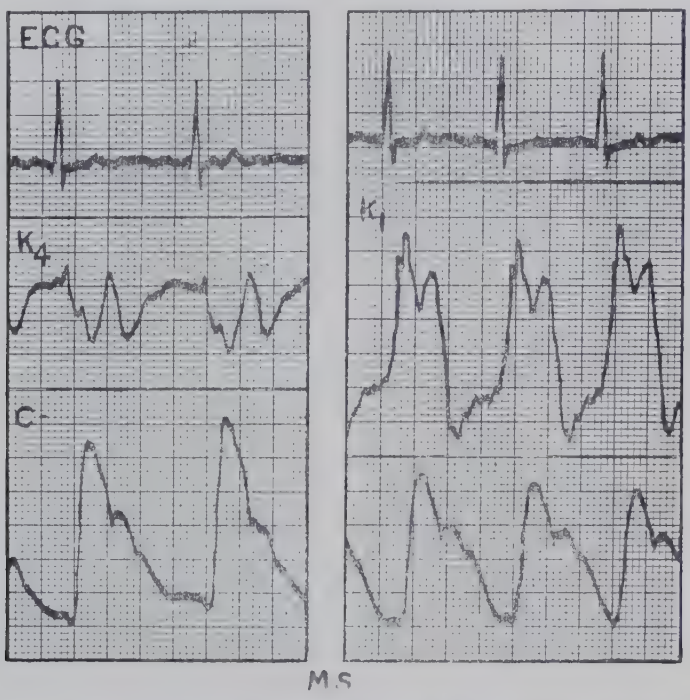

FIG. 4.—Kinetocardiographic tracing from a patient with "pure" mitral stenosis. Note the marked, early, outward systolic movement of the parasternal region of the chest. In addition, the parasternal region of the chest is sustained outward during the entire ejection phase, in contrast to retraction during ejection in normal subjects.

sible if the chest wall is in effect able to differentiate the displacement movements of the heart twice, thus registering force patterns instead of displacement. The fact that several phenomena are being registered in the kinetocardiogram does not appear to limit its value, but theoretically should add to its over-all usefulness not only by giving information about possible movements of the heart and the effects of ejection but by serving as a semidirect technic in recording the forces associated with myocardial contraction and relaxation. Kinetocardiograms cannot be considered as posteroanterior ballistocardiograms, since the tracings differ in configuration from point to point over the precordium. In addition, the marked similarity of simultaneous electrokymographic and

kinetocardiographic tracings taken in the region of the apex is evidence that the kinetocardiograms are not posteroanterior ballistocardiograms. In summary, the tracings appear to be related to *1)* movements and shape changes of the heart, *2)* chest volume changes or shifts in the intrathoracic blood, and *3)* possible forces associated with myocardial contraction and relaxation.

CLINICAL APPLICATION

Clinical studies of records obtained with this technic are few, so that the full scope of its clinical application is unknown at the

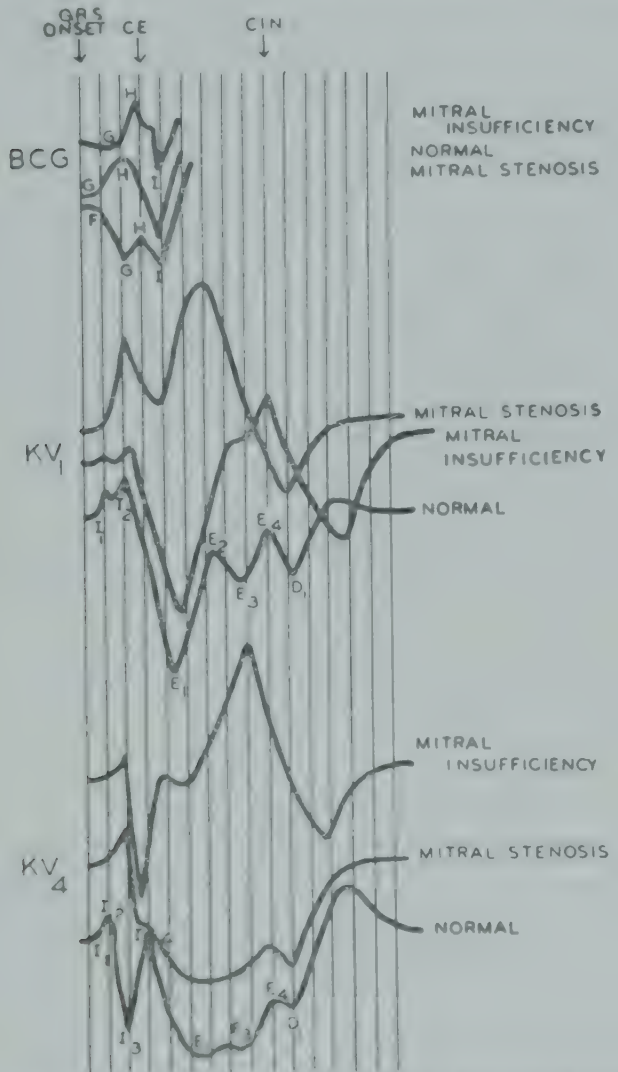

FIG. 5.—Drawing illustrating differences in the kinetocardiographic tracings from patients with mitral stenosis and mitral insufficiency, as compared to normal subjects. Note the marked dissimilarity.

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present time. It does appear that significant information can be obtained with this technic in two respects:

1) As the low frequency movements in the precordial tracings are probably primarily related to the motions of the heart and to cardiac forces, it is likely that information can be obtained about the mechanism of ventricular contraction and relaxation. Since

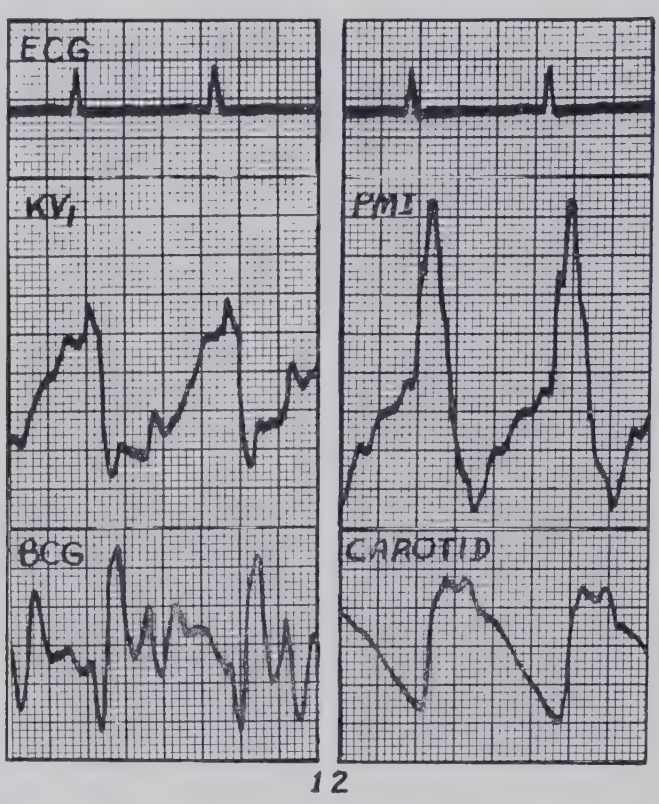

FIG. 6.—Kinetocardiographic tracings (K_1 and PMI areas) from a patient with aortic insufficiency. Note the marked exaggeration in the apex thrust, followed by a marked retraction as ejection occurs. The pattern is significantly different from those of mitral stenosis and mitral insufficiency.

the cardiac movements, and probably the cardiovascular forces, are primarily related to the structure of the muscle bundles, changes in the anatomic structure, such as hypertrophy or destruction of myocardium, are reflected by changes in the kinetocardiograms. Disturbances in the conduction process similarly distort the sequence of ventricular contraction and relaxation, and, in turn, alter the precordial movements (7). The entire field of the mechanisms and the sequence of ventricular contraction and relaxation in various cardiac diseases is essentially unexplored. Thus the kinetocardiogram appears to offer an easily applied technic by which these basic physiologic processes may be studied.

Consistent alterations in the kinetocardiograms have now been found to occur in various cardiovascular disease states (1, 6, 7, 9). Figure 4 is an example of the kinetocardiographic pattern in patients with mitral stenosis (6). The configuration differs appreciably from that noted in normal subjects (Fig. 2). In addition, different types of patterns have been noted in patients with mitral insufficiency. Figure 5 illustrates the differences in the

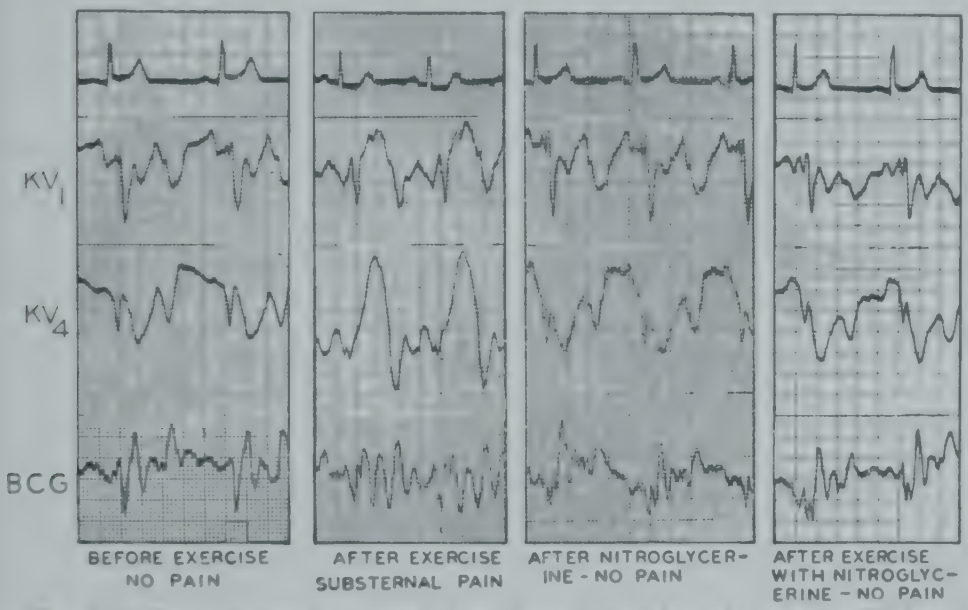

FIG. 7.—Simultaneous tracings taken from the region of the apex (K_4) and the left parasternal region of the chest (K_1) in a patient with angina pectoris. Note the marked bulging of the precordium after exercise during pain, which reverted to the original pattern upon rest. This marked bulging was prevented by nitroglycerin taken just before the subsequent exercise. The marked outward bulge in tracing after exercise during pain suggests aneurysm of the ventricle as the result of an ischemic process associated with the pain.

kinetocardiographic tracings from patients with mitral stenosis and mitral insufficiency, as contrasted to normal subjects (9). Because the differences are sharp, this technic apparently offers an additional means of evaluating the degree of mitral insufficiency in the presence of mitral stenosis. Similarly consistent changes (markedly different from normal) have been noted in aortic insufficiency (1) (Fig. 6) and aortic stenosis. Marked differences from the tracings of normal subjects frequently occur in patients with coronary artery disease. Tracings from the apical region in a patient before and during anginal pain, and after the administration of nitroglycerin are shown in Figure 7; note that the tracing tends to return to normal after the administration of nitroglycerin.

In summary, it seems likely that kinetocardiographic tracings may be a means for studying the processes of ventricular contraction and relaxation. In addition, it appears that certain configurational changes may be of importance in the diagnosis and evaluation of cardiovascular disease. The relative importance of cardiac forces, of displacement of the chest wall by heart movements, and of volume changes in the genesis of the kinetocardiogram is uncertain at the present time.

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NITROUS OXIDE METHOD FOR DETECTING SMALL INTRACARDIAC SHUNTS

RICHARD GORLIN

It is not always possible to detect the presence of small left-to-right shunts by the oxygen admixture principle. Morrow (1) has described a method originally devised by Callaway which is highly useful for the diagnosis of the smaller shunts. This method employs 15% nitrous oxide inhalation. During the early minutes of this inhalation, all the tissues of the body are rapidly taking up this foreign gas and little appears in the venous blood. As a result, a more striking difference exists between arterial and venous gas concentration than exists for oxygen (Table 3). Normally, an inte-

TABLE 3. Detection of Small Shunts by Nitrous Oxide Technic

	Oxygen Admixture, Vol %	Nitrous Oxide (1 Min), Vol %
Normal		
Superior vena cava	13.4	0.4
Right atrium	14.3	0.4
Difference	0.9	0
Femoral artery	18.4	3.4
Morrow ratio*	$\frac{14.3 - 13.4}{18.4 - 13.4} = 18\%$	$\frac{0}{3.4 - 0.4}$
Atrial Septal Defect		
Superior vena cava	13.4	0.4
Right atrium	14.3	2.1
Difference	0.9	1.7
Femoral artery	18.4	3.4
Morrow ratio*	18%	$\frac{2.1 - 0.4}{3.4 - 0.4} = 54\%$

* Morrow ratio: $\frac{\text{Right atrium} - \text{superior vena cava}}{\text{Femoral artery} - \text{superior vena cava}} = \%$

grated arterial sample drawn continuously during the first minute of gas inhalation will contain about 3 vol % nitrous oxide. The normal mixed venous sample drawn at the same time will contain much less, usually less than 1 vol %. With a left-to-right shunt, passage of blood containing 3 vol % to an area with less than ½ this amount will cause a marked rise in nitrous oxide concentration in venous blood downstream from the shunt. Morrow recommends the use of a double lumen catheter, with one orifice before the suspected shunt and the other orifice at or beyond. Simultaneous samples drawn from a systemic artery and the 2 venous sites should

give good control. Usually, nitrous oxide concentration beyond the shunt will exceed that before the shunt by about 1.5 vol %. This test is helpful as a screening procedure for all small left-to-right shunts if it is first performed with the catheter tip in the main pulmonary artery. The shunt may then be localized by repetition across each pair of chambers.

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CORONARY BLOOD FLOW

RICHARD GORLIN

In 1945, Kety and Schmidt (5, 6) introduced the technic of measuring cerebral blood flow by inhalation of a metabolically inactive foreign gas, 15% nitrous oxide. The method was soon adapted for measuring the coronary blood flow (1, 3). N_2O dissolves in heart muscle tissue in a nearly 1:1 ratio with the blood. The muscle concentration of N_2O is determined by measuring the venous concentration after 10 min of inhalation, because at this point the tissues are saturated and myocardial and venous blood are in equilibrium. This value is multiplied by 100 to give concentration in vol/100 g of muscle. An arteriovenous difference is measured either during the 10 min period of N_2O tissue saturation or during the desaturation period following cessation of N_2O breathing. The 10 min integrated arteriovenous difference is divided into the N_2O uptake/100 g to derive the flow.

In order to obtain coronary venous blood, it is necessary to locate the catheter in the coronary sinus. Most workers agree that blood samples from this site are derived primarily from the left ventricular muscle (1, 3). The technic of placing the catheter in the coronary sinus is highly variable and the percentage of deliberate success ranges from 50–90%. The sinus entrance is located on the posterior wall of the right atrium near the eustachian ridge just above the inlet of the inferior vena cava. The catheter used is a special bird's eye tipped catheter (1, 3) preformed by heat hardening, with a 110° angle to the last 7 cm. I prefer to have the patient placed in the left lateral position. Once inserted into the heart, the catheter is advanced from the middle of the right atrium toward the inferior vena cava. The tip should be twisted so as to point in a posterior and medial direction, i.e., oriented away from the tricuspid valve and pulmonary outflow tract. The catheter is then repeatedly moved through the plane described. The terminal portion of the catheter will eventually pass along the posterior border of the heart toward the left atrium. In the anterior view, the catheter will usually appear to be headed toward the pulmonary artery. The catheter takes on a characteristic movement in which the entire tip moves as a unit cephalad and caudad in a plane perpendicular to the catheter itself. The catheter is now within the sinus and is being carried up and down with each systole. The catheter should be advanced only as far as is necessary for stability, and a

check should be made to see if blood may be withdrawn freely. The blood from this area has the lowest oxygen content in the body, and consequently the dark color is a clue to the proper placement of the catheter.

The nitrous oxide inhalation may now be started. The sample for the arteriovenous difference is drawn either during saturation or desaturation, the latter perhaps being preferable because errors in gas inhalation are eliminated (2). We have found it advisable to take blood samples at multiple intermittent points, with washing out in between in order to prevent clotting of the catheter tip. Goodale and Hackel (2), on the other hand, believe the greatest accuracy in measuring total arteriovenous difference to be achieved

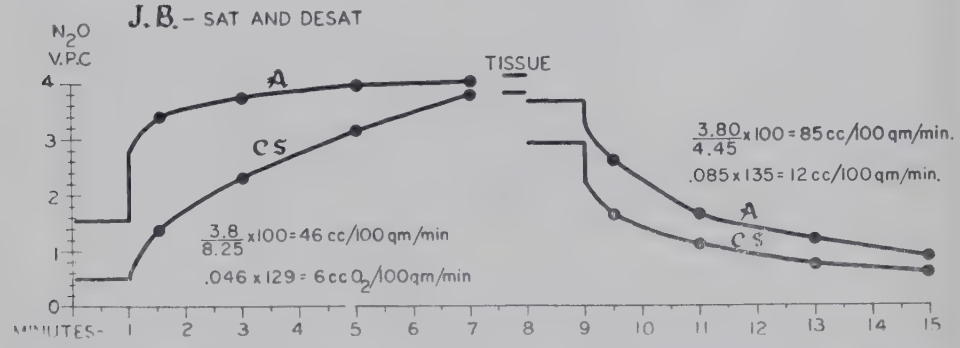

FIG. 1.—Effect of nitroglycerin on coronary blood flow and oxygen consumption.

by obtaining continuously drawn multiple integrated samples. By either method it is important to draw a carefully integrated sample throughout the first minute of gas saturation or desaturation. The organ concentration of gas is assumed to be the same as the coronary venous concentration after 10 min of saturation. This blood sample is drawn either immediately following the last pair of samples for a saturation arteriovenous difference or immediately before the first samples of a desaturation curve.

A minor source of error lies in the time lag between coronary sinus and arterial sampling due to the catheter length. This 2–3 sec lag in time is important only in the first 3 min of flow determination and particularly in the initial integrated sample. This lag may be obviated by sampling the arterial blood through a similar length of catheter tubing or, having previously determined the time, by discarding the sample drawn through the period of lag and continuing sampling the same extra seconds beyond the stated period for sampling.

The samples are analyzed for N_2O (6) as described below, and the points are plotted on a graph as shown in Figure 1. A smooth

curve is drawn through the points and the area is integrated planimetrically to give the total arteriovenous difference. Points which lie far off a smooth curve must be rechecked. If inconsistent values occur, especially in the first 3 min of a saturation or a desaturation curve, it is best discarded.

Coronary flow (CF) is calculated as follows:

$$CF = \frac{N_2O \text{ conc in organ (coronary sinus)}}{\int (A - V_{N_2O})dt}$$

where $(A - V_{N_2O})dt$ = nitrous oxide arteriovenous difference integrated with respect to time.

Flows are expressed in cc/100 g min. Normal values lie between 60 and 100 cc, and vary according to cardiac work, cardiac rate, neurohumeral factors, etc., operating at the same time as the flow determination (4). The method is most valid when repeated on the same patient under varying conditions, and least valid when groups of patients or diseases are compared.

PRINCIPLE OF NITROUS OXIDE ANALYSIS

The principle of measurement is essentially as follows: Oxygen and carbon dioxide are removed in the first step of the analysis. The remaining gas free in the extraction chamber contains nitrogen and nitrous oxide. A blank blood sample is run prior to nitrous oxide inhalation. The partial pressure of this sample represents nitrogen and other inert gas. When this partial pressure is subtracted from the pressure of the samples during nitrous oxide inhalation, the remaining partial pressure represents nitrous oxide.

Equipment and Materials

1) Van Slyke manometric apparatus; *2)* two 2 cc pipets with stopcocks; *3)* one 30 cc syringe to contain water and caprylic alcohol; *4)* one 10 cc syringe for oxygen absorber; *5)* one interval timer.

Reagents

1) Capryl alcohol and distilled water. Add 25 cc distilled water and 5 cc capryl alcohol to the Van Slyke extraction chamber. Extract dissolved gases over two 3 min periods and transfer to 30 cc syringe.

2) Oxygen and carbon dioxide absorber. To 2 g of a 10:1 mixture of sodium hydrosulfite and sodium anthraquinone β-sulfonate, add 10 cc of 2N potassium hydroxide. Filter through cotton into Van Slyke extraction chamber and extract gases over two 3 min intervals. Transfer to 10 cc syringe. The syringe should have previously contained 3 cc of mineral oil. Invert syringe and eject all

air bubbles from oil and syringe. The oil will float on top of the absorber and make it possible to read the graduations on the syringe. The absorber will absorb oxygen and carbon dioxide, leaving nitrogen and nitrous oxide in the chamber.

PROCEDURE

1) Transfer 2 cc of blood from pipet into extraction chamber. *2*) Invert 30 cc water and capryl alcohol syringe, plunger at bottom, and fill syringe capillary with capryl alcohol. *3*) Plunge syringe capillary below mercury in cup and deliver 5 cc water. *4*) Deliver 2 cc absorber into extraction chamber, and seal with mercury. *5*) Bring solution down to 50 cc mark and extract for 3 min. *6*) Take P_1 reading at 2 ml mark and record temperature. *7*) Eject the solution from the chamber and begin the next analysis.

Calculation

Just before the blood analysis is started, a blank analysis is made, using 2 cc of deaerated water instead of blood. In addition to the water blank, a blood blank value (X) must be obtained by analysis of a sample obtained just before the N_2O inhalation begins. This is part of the correction for nitrogen and other inert gases. The formula for calculating nitrous oxide concentration in vol % is:

$$F'N_2O[ra - (ro + Cwa)] - F'N_2O[rx - (ro + Cwx)]$$

where $F'N_2O$ of blood sample is determined by using the temperature of blood sample; $F'N_2O$ of X factor, by using the temperature of blood blank; ro is the reading of water blank in mm Hg; rx, the reading of blood blank in mm Hg; ra, the reading of blood sample in mm Hg; $Cwa = \Delta Pw(ta - to)$; $Cwx = \Delta Pw(tx - to)$; to is the temperature of water blank; tx the temperature of blood blank; ta, the temperature of blood sample; and ΔPw is determined by to.

Table 4 supplies the factors needed in the formula for calculating the concentration of N_2O. The entire procedure may be performed on ice aliquots of blood. The volumes of the reagents are reduced accordingly and the factors in Table 4 doubled.

Comment by Walter T. Goodale

The Kety concept of measuring organ blood flow is best suited to heart muscle flow for several reasons: *1*) the myocardium provides homogeneous tissue without contaminating venous drainage from noncardiac tissue; *2*) it offers the proper range of flow to allow for accurately measurable nitrous oxide arteriovenous differences; and *3*) its excellent capillary–

TABLE 4.—Factors for Nitrous Oxide Determinations

Temperature, °C	$F'N_2O$	ΔPw	Temperature, °C	$F'N_2O$	ΔPw
20.0	0.1456	1.1	25.6	0.1415	1.5
.1	.1455		.7	.1414	
.2	.1455		.8	.1414	
.3	.1454		.9	.1413	
.4	.1453		26.0	.1412	1.5
20.5	.1452	1.2	.1	.1411	
.6	.1452		.2	.1411	
.7	.1451		.3	.1410	
.8	.1450		.4	.1409	
.9	.1450		26.5	.1408	1.6
21.0	.1449	1.2	.6	.1408	
.1	.1449		.7	.1407	
.2	.1448		.8	.1406	
.3	.1447		.9	.1406	
.4	.1446		27.0	.1405	1.6
21.5	.1445	1.2	.1	.1404	
.6	.1445		.2	.1404	
.7	.1444		.3	.1403	
.8	.1443		.4	.1402	
.9	.1442		27.5	.1401	1.7
22.0	.1441	1.2	.6	.1401	
.1	.1440		.7	.1400	
.2	.1440		.8	.1399	
.3	.1439		.9	.1399	
.4	.1438		28.0	.1398	1.7
22.5	.1437	1.3	.1	.1397	
.6	.1437		.2	.1396	
.7	.1436		.3	.1396	
.8	.1435		.4	.1395	
.9	.1435		28.5	.1394	1.7
23.0	.1434	1.3	.6	.1393	
.1	.1433		.7	.1392	
.2	.1433		.8	.1392	
.3	.1432		.9	.1391	
.4	.1431		29.0	.1390	1.7
23.5	.1430	1.4	.1	.1389	
.6	.1430		.2	.1389	
.7	.1429		.3	.1388	
.8	.1428		.4	.1387	
.9	.1428		29.5	.1386	1.7
24.0	.1427	1.4	.6	.1386	
.1	.1427		.7	.1385	
.2	.1426		.8	.1385	
.3	.1426		.9	.1384	
.4	.1425		30.0	.1383	1.8
24.5	.1424	1.4	.1	.1382	
.6	.1423		.2	.1382	
.7	.1422		.3	.1381	
.8	.1422		.4	.1380	
.9	.1421		30.5	.1379	1.8
25.0	.1420	1.4	.6	.1379	
.1	.1419		.7	.1378	
.2	.1418		.8	.1377	
.3	.1418		.9	.1377	
.4	.1417		31.0	.1376	1.8
25.5	.1416	1.5	.1	.1375	

tissue diffusion, with several capillaries surrounding each muscle bundle and without sarcolemmal sheath to impede diffusion.

Several limitations to the nitrous oxide method as applied to coronary blood flow should be mentioned:

1) Human heart muscle contains more fat and more variable amounts of fat, than canine heart muscle, requiring a solubility factor of 1.1 or more, rather than 1.0. Nitrous oxide is more soluble in fat than in muscle, and the blood flow to fat depots is probably much lower. This introduces a "contaminating" factor in the coronary flow curves of some fat people. The initial, rapidly changing arteriovenous differences are relatively unaffected, but the later samples sometimes show lack of equilibration between arterial and venous nitrous oxide levels. This failure to equilibrate may sometimes be due to nitrous oxide transfer to or from the epicardium or endocardium, as suggested by Gregg, especially if there is pericardial fibrosis or if there are scarred areas communicating with the mediastinal venous system. Errors in calculations of the type reviewed by Saperstein and Odgen are thus introduced, although to a lesser extent than in cerebral blood flow measurements. However, the desaturation technic seems less likely to produce failure of arteriovenous equilibration than the original saturation technic.

2) The method only measures mean flow over 6–10 min, and is often grossly distorted unless the flow remains constant for this period. It will not measure flows accurately at more than 100 cc/100 g/min, since arteriovenous equilibration then occurs almost immediately.

3) Like all regional methods, it fails to distinguish between functional myocardial tissue flow and nonfunctional flow through coronary arteriovenous anastomoses, scarred areas or areas of previous surgery for coronary or pericardial disease. This limits its value in assessing improvement following such operations.

4) In the open chest or in the operative preparation, there are far better methods without the limitations noted.

The nitrous oxide technic, however, is the only practical method available for studies in man. It accurately reflects changes in flow, and provides a fair standard of comparison between individuals and in pathologic conditions on a basis of flow per 100 g of muscle. However, inability to calculate total flow prevents accurate estimations of absolute mechanical efficiency, although relative changes in efficiency in the same patient can still be measured. Finally, flow measurements are even more important in the interpretation of changes in coronary arteriovenous differences reflecting metabolic exchange, which after all constitutes the major purpose of coronary blood flow.

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SECTION III

Methods for the Study of Human Leukocytes

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INTRODUCTION

THE HUMAN LEUKOCYTE has the potentiality of being an excellent cell for metabolic studies because it is readily removed from the body without serious operative procedure and has the desirable property of being in suspension as single cells. The obstacle to this study has been freeing the leukocytes of contamination with red cells, platelets and other blood constituents. This has been overcome to a degree by the sedimentation procedures that lower the red cell and platelet volume to the point where their activity constitutes a small part of the functions studied. By mathematic procedures the role of each cell can be assayed.

The physiologic and biochemical function of the leukocyte is not known. Its scavenger role in bacterial defense has been extensively studied on an anatomic basis, but little chemical information is available to characterize this function. This cell may be the ideal one for the study of cellular metabolic activities in man, since observation *in vitro* may give information that is not obtained by study of the body fluids which are subjected to compensatory mechanisms of many highly specialized cell systems. Whether the specialized functions of the leukocyte will overshadow its general cellular metabolic functions must await further studies. Evidence is available to indicate that the leukocyte may carry the mark of the metabolic disturbances of the body as a whole (2, 3).

The aspects of the studies of leukocytes presented here are varied, but limited in number. Representative technics covering a wide field are presented with no attempt to be inclusive. Cytochemical technics are completely omitted because reviews in this

area are available (1). The selection of technics was conditioned by an attempt to give a kaleidoscopic picture which would serve as a springboard for the investigator to go into other areas of application than those at present being used by the authors.

In vitro as well as *in vivo* technics are presented. The *in vitro* technics vary from the measurement of physiologic and biochemical function of surviving cells to the strict chemical determination of cell constituents. The tissue culture technic of human leukocytes was included for its many uses in the study of surviving cells. The *in vivo* technic included in this review is the cover-slip technic, which because of its simplicity makes possible numerous morphologic and cytochemical studies of the inflammatory reaction.

—Samuel P. Martin.

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SEDIMENTATION TECHNICS

JAMES L. TULLIS *and* PHILIPPA BAUDANZA, *Harvard University*

Leukocytes can be obtained for *in vitro* study by sedimentation technics (5). Only standard equipment and simple reagents are required. The methods take advantage of the rapid settling of red cells, leaving a supernatant plasma in which the white cells are largely undiminished. This plasma suspension later can be concentrated and washed in an ordinary laboratory centrifuge (8).

Red cell sedimentation is due chiefly to rouleau formation (3). Aggregated red cells follow Stokes' law. Fibrinogen is the protein largely responsible for such rouleaux (4). Other asymmetric molecules can be added artificially to whole blood to produce the same effect (6). The action of such agents can be reversed by albumin and glycine. Sedimentation has the disadvantage of: *1*) sedimentation time, during which metabolic degradation of the leukocyte occurs, and *2*) added rouleau agent, which is later difficult to wash from the surface of the cells.

ROULEAU REAGENT

This may be: *a*) fibrinogen (both bovine and human), *b*) dextran, *c*) dextrin, *d*) glutamyl peptide, *e*) polyglucose, *f*) Periston (polyvinylpyrrolidone), or *g*) gamma globulin. The choice of reagent depends on the experiment for which the leukocytes are desired. Dextrans and polyglucose may interfere with certain carbohydrate studies. Gamma globulin and Periston may interfere with antigen studies. Fibrinogen usually contains added citrate, which may interfere with Krebs cycle studies. Three physical variables—molecular characteristics, concentration of rouleau reagent, and temperature—must be considered in the sedimentation.

Molecular Characteristics

Size.—Synthetic rouleau agents have a population of mixed molecular sizes. They can be fractionated to improve homogeneity but they cannot be purified completely. The most representative physical measurement for size distribution is *intrinsic viscosity*, which for dextran is 0.42–0.46, and for dextrin is 0.35–0.39. The relation of molecular size to optimal red cell packing is given in Table 5.

Weight.—Many of the synthetic agents can be obtained in either

TABLE 5.—Relation of Molecular Size of Synthetic Rouleau Agents to Hematocrit

Dextrin, No.	Viscosity	Reducing Power, MH mg/g	Final Conc, %	Hematocrit, in Min 10	20	30	40	50	60
3802-21B	0.385	0.6	0.8	91	77	70	68	67	67
			1.5	90	62	53	50	48	48
3797-5C	0.370	1.9	0.8	96	88	80	78	73	72
			1.5	83	68	60	59	57	55
3802-21C	0.362	0.9	0.8	97	88	81	78	76	75
			1.5	90	72	59	53	51	50
3797-4C	0.350	1.6	0.8	92	81	75	71	69	68
			1.5	82	60	54	51	49	48
3802-21D	0.266	1.1	0.8	97	92	88	85	82	79
			1.5	98	80	73	69	69	65

TABLE 6.—Sedimentation with Various Concentrations of Synthetic Rouleau Agents

Concentration, %	Time, Min 15	30	40	60	90
Polyglucose Sedimentation					
High molecular weight					
1.0	70	53	45	44	44
1.5	90	53	45	44	44
2.0	99	74	48	44	44
Medium molecular weight					
1.0	90	60	48	46	43
1.5	91	64	52	43	43
2.0	91	53	45	43	43
Low molecular weight					
1.0	98	92	88	84	76
1.5	99	97	93	90	84
2.0	99	98	95	93	87
Polyvinylpyrrolidone (Periston) Sedimentation					
PVP (K-90)					
1.0	84	61	56	55	
2.0	98	90	85	80	
3.0	100	92	87	80	
PVP (K-60)					
1.0	70	60	56	55	
2.0	95	87	78	72	
3.0	91	82	76	69	

high, medium or low molecular weight. The higher molecular weight fractions yield more rapid sedimentation (Table 6).

Charge.—A few synthetic reagents may be obtained with varying degrees of molecular charge. Elimination of charge increases sedimentation effect up to approximately 50% saturation of the sedimentation molecule. Elimination of charge beyond 50% negates this beneficial activity (Table 7).

TABLE 7.—Effect of Synthetic Peptides on Sedimentation*

Glutamyl Peptide	Molecular Wt	Hematocrit, Min					
		10	20	30	40	50	60
B-260 (plain)	12,400	98	96	95	90	88	85
B-260 (59% methylated)	12,400	99	83	58	53	53	52
B-326 (87% methylated)	12,600	96	88	76	71	67	60
Control (final hmt 57)	—	100	96	93	85	81	80

* Data were obtained by mixing 3.5% peptide with aliquots of blood from a single donor (R00283N), dilution 1:6, to give final peptide concentration of 0.6%. Temperature, 23° C.

Secondary (Nonrouleau) Sedimentation

The proximity of individual red cells to each other is critical for satisfactory rouleau formation. Once the red cell concentration is reduced to a level of less than 10,000/mm^3 by a satisfactory sedimentation, the remaining cells are too far apart properly to aggregate and sediment according to Stokes' law. Thus, a repeat sedimentation (of the rouleau type) will not improve the white:red cell ratio of roughly 1:1. A secondary spontaneous sedimentation will greatly improve this ratio and yield high concentrations of white cells if viability is not critical. The secondary sedimentation requires 90 min at room temperature in a liquid medium which has been rendered nonviscid by the elimination of plasma proteins and electrolytically unbalanced by high dextrose (5 g/100 cc) and low salt (0.05M or less) concentrations. This sedimentation is most effective in tubes which are more than 15 cm long and less than 1 cm wide.

General Considerations

If viable leukocytes, free of surface damage or enzyme release are desired, all of the above sedimentations must adhere to the general requirements for white cell study:

1) Nonwettable glassware. All metal and glass surfaces should be protected by a layer of nonwettable reagent (6).

2) Ion exchange resin. Decalcification should be substituted for anticoagulants (citrate, EDTA, oxalate) as the latter will complex with divalent cations and thus interfere with normal intracellular enzymic activity (7).

3) Glucose should be added to the sedimentation system to support metabolism during the sedimentation time. This is particularly important when temperatures above 12° C are used.

4) Desoxyribonuclease should be added if the experimental con-

ditions require use of the sedimented leukocytes for 24 hr or more. This enzyme in a concentration of 5 units* ml is nontoxic to leukocytes and will eliminate nucleoprotein precipitate in the medium.

CONCENTRATION OF ROULEAU REAGENT

If the use for which leukocytes are needed makes advisable minimal concentrations of rouleau agents, a lower yield and a longer time will ensue. The parameters of time versus concentration versus optimal red cell packing are illustrated in the case of fibrinogen in Figure 1.

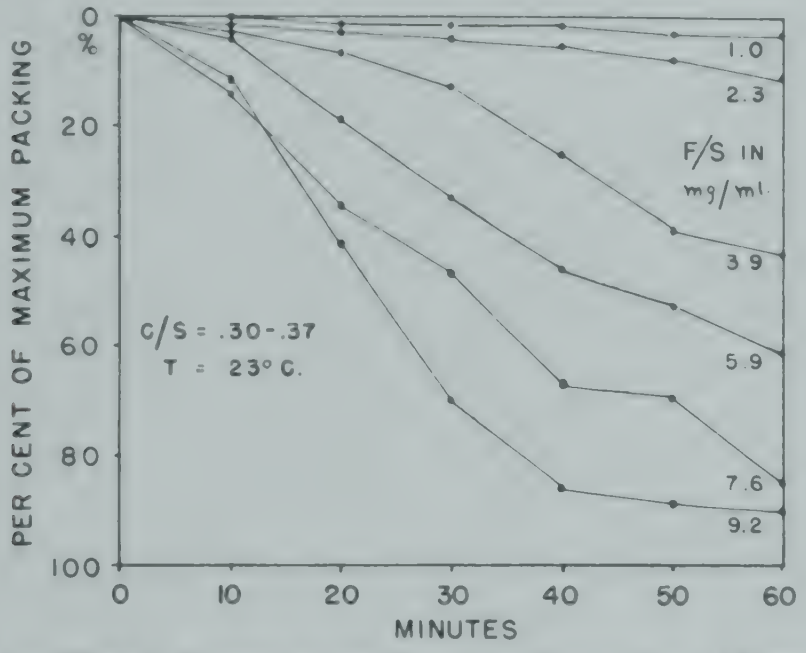

FIG. 1. Effect of fibrinogen on erythrocyte sedimentation. (Figures 1-3 have been reproduced by permission of the publisher, J. Lab. & Clin. Med. 36:29–39, 1950, and the authors [2].)

TABLE 8.—REAGENTS CONCENTRATION AND ERYTHROCYTE CONCENTRATION†

AGENT	FINAL CONC	TIME, MIN	HEMATOCRIT
Fibrinogen	0.92	35	57.5
Dextran (intrinsic viscosity 0.46)	0.8	35	47.5
Dextrin (α-amylase pectin)‡	1.5	35	45.0
Glutamyl peptide§	0.6	35	32.0
Polyglucose	1.0	45	45.0
Periston	2.5	40	50.0
Gamma globulin, human	7.0	60	56.5

† All sedimentations carried out by standard technics using a Wintrobe hematocrit tube, 21° C.

‡ Lot No. 3247.77B, Northern Regional Laboratories.

§ Molecular weight 23,000, prepared by Dr. Max Bovarnik, Veterans Administration Hospital, Brooklyn 9, N. Y.

*Varidase (Lederle).

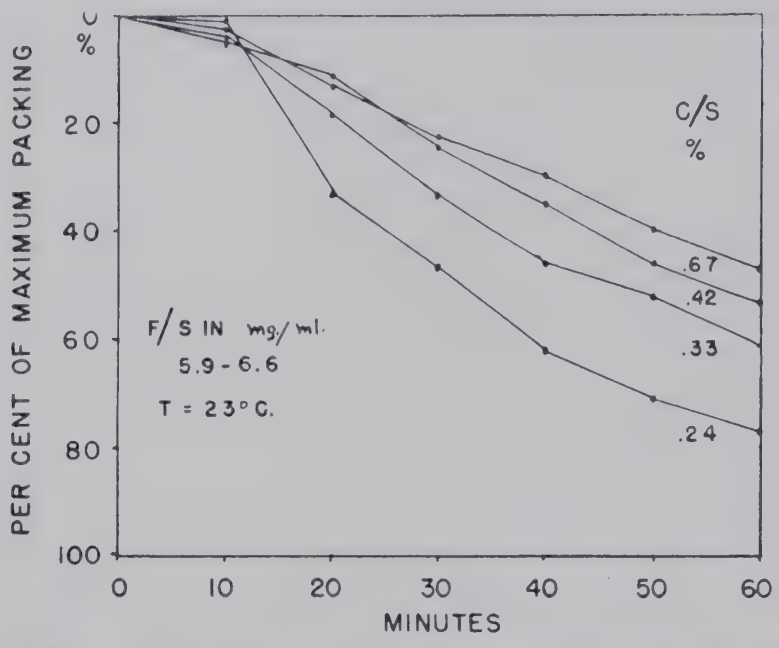

FIG. 2.—Effect of cell-supernatant ration on erythrocyte sedimentation (2).

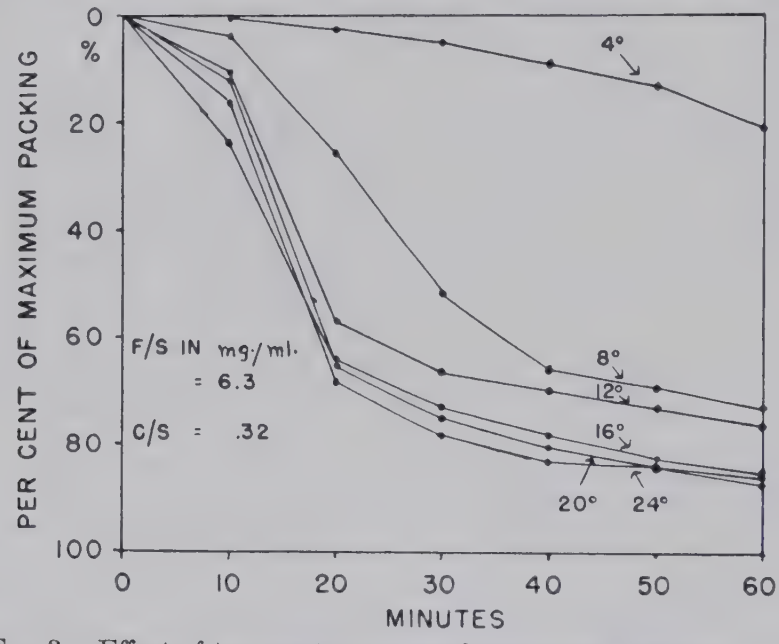

FIG. 3.—Effect of temperature on erythrocyte sedimentation (2).

The optimal concentration varies for the different reagents (Table 8). The ratio of red cells to liquid phase is equally important. This is due to reciprocal displacement of fluid in a direction opposite to the fall of the red cells. The absence of spontaneous sedimentation in polycythemic blood is an example of such interference. The optimal ratio is about 0.3, with a range of 0.24–0.42 (1, 2) (Fig. 2).

TEMPERATURE

Satisfactory rouleau formation and sedimentation will occur between 12° and 25° C. At lower temperatures, the process is retarded by slower molecular movement and increased viscosity. Thus, the optimal temperature for maintenance of leukocyte metabolism (4°) conflicts with the optimal temperature for sedimentation. If the leukocytes are desired for carbohydrate metabolism study 8°–12° is recommended (Fig. 3). This temperature will require longer sedimentation and will yield fewer white cells but is within the practical range for standard experiments.

METHOD

The following method is suggested, subject to variation depending on choice of rouleau agent, temperature, etc. The volumes are for recovery of leukocytes from a 500 ml phlebotomy, but may be reduced to any desired amount. All data are for human blood.

Dissolve 4.0 g dextran (intrinsic viscosity 0.40–0.46), 3.0 g dextrose and 0.85 g sodium chloride in 100 ml distilled water. Add 100 ml dextran solution to 500 ml whole blood. Invert bottle and suspend for 30 min at room temperature (20°–25° C). Draw off sedimented red cells. Concentrate leukocytes from residual plasma. Wash. Resuspend in 5% dextrose, if desired.

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METHODS FOR THE STUDY OF SURVIVING LEUKOCYTES

A. PREPARATION OF CELL SUSPENSION*

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IN THE STUDY of surviving leukocytes, as with tissue culture studies, scrupulous care of glassware is important. Its importance is best recognized in the ability to obtain consistent and reproducible data with narrower margins of normal variation. It is most noticeable in experiments in which cells are maintained for 1 hr or more. The glassware should be free of pyrogens and detergents, and gives most consistent results when all surfaces have been treated with an agent to render them nonwettable (1). The following is an outline of procedure for the care of glassware, syringes and needles.

1) All glassware is washed in a mild detergent, rinsed 8 times with tap water, and 8 times with distilled water. The hubs and crevices in needles are carefully cleaned.

2) The glassware is placed in a solution of 15% KOH in 50% alcohol for 24 hr. After removal, it is washed again in tap and distilled water, as above.

3) The glassware is baked for 2 hr at 180° C in an oven.

4) The glassware is placed in a paper box, allowed to sit for 6 hr at room temperature, then exposed for 15 sec to the vapor of Dri-film (SC 77).† This procedure is carried out in a chemical hood. The vaporization is accomplished by placing 4 ml silicone in a flask with inflow and outflow tubes. Air is passed through the flask, and the vapor discharged into the box containing the glassware.

5) The siliconed glassware is rinsed 6 times with distilled water, packed and autoclaved. During this process, complete siliconing is ascertained by observing the movement of the surface of the wash water over the surface of the glass to detect surface irregularities.

6) Steps 1–5 are repeated after each use.

7) The needles are cleansed, dried and treated with a solution of 2 parts of Dribake (SR-02)† to 5 parts toluene or xylene. This covers the surfaces exposed to blood. After using, the needles are immediately placed in water, washed carefully and dried. The

* This work was supported in part by a grant (G3476) from the National Institutes of Health, The Duke Research Council, and The Baxter Laboratories, Inc.

† General Electric Company, Silicone Products Department, Waterford, N. Y.

needles can be used 20 to 30 times after 1 treatment with Dribake.

In the study of the functions of surviving cells, it is extremely important that the blood be handled with a minimum of physical and chemical trauma. In addition to the glassware, there are other important factors. The venipuncture is made with a large needle (18 gage), inserted with a rapid clean puncture, since tissue juices or blood clots markedly alter glycolysis and respiration, as well as induce early irreversible clumping of the cells. A series of agents were tried as anticoagulants; 3 were useful, and heparin* was preferable (0.02 mg/ml). Drawing blood through an ion exchange column, or the use of ethylenediaminetetraacetate, gave satisfactory results. During the process of drawing the blood and subsequent mixing with the anticoagulant, bubbles and violent shaking must be avoided. This should also be considered in all subsequent pipeting operations. In addition, high centrifugal forces will completely disrupt normal cell function.

Dextran sedimentation was used throughout most of the studies, because it was the only agent which maintained the necessary degree of the cell's chemical integrity. Various dextrans have been tried; many lots of dextran contained an agent which was injurious to leukocytes, but occasionally this agent could be removed by passing the final solution through a charcoal column. Dextrans prepared by Pharmacia, Lot 834109, or Laros, Grade III (certain lots) were the most satisfactory.

Two methods of collecting blood were used: *1*) bleeding into a Fenwal blood pack containing an ion exchange resin or heparin, and *2*) use of siliconed 20 ml syringes.

1 ml of 5% dextran dissolved in buffered saline for each 5 ml of blood was used for sedimentation. This sedimentation procedure could be carried out in test tubes with 10 ml of blood or in the plastic blood pack. If test tubes were used, they were stoppered with a rubber stopper; the supernatant plasma was removed as it cleared in order to increase the yield. If the pack was used, the plasma was expressed after 30 min of sedimentation into a 250 ml centrifuge tube, the mixture was centrifuged at 50*g* for 15 min, the plasma and platelets were decanted, and the leukocytes were resuspended in a modification of Hanks' solution.† The solution was prepared by diluting 10 ml stock solution (containing in 1 liter 75 g of NaCl, 7.5 g of KCl, 1.0 g of Na_2HPO_4, 1.2 g of KH_2PO_4 and 5 g K_2HPO_4) with 7.0 ml of another solution containing 10 g of Na_2HPO_4 per liter and brought to a final volume of 100 ml.

* Liquaemin sodium, Organon Inc., Orange, N. J.
† Devised by Dr. J. L. Tullis.

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B. RESPIRATION

GORDON R. McKINNEY, *University of West Virginia, and* SAMUEL P. MARTIN

Respiration of surviving cells was measured by the direct method of Warburg (1).

1) Cell suspension of 60,000 cells per mm^3 were prepared as already described.

2) 1.5 ml aliquots of cell suspension were added to a small volume Warburg flask containing 1.5 ml of the modified Hanks' solution. Material to be studied was added either to the vessel or to the side arm.

3) The vessels were aerated and prepared in the usual manner.

4) Readings were taken at 10 min intervals.

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C. GLYCOLYSIS

SAMUEL P. MARTIN, R. GREEN *and* GORDON R. McKINNEY

Aliquots of the surviving leukocyte preparations were studied for glucose utilization and lactic acid production. The determinations were carried out on much smaller quantities of cells than those used to study respiration, 1 ml aliquots containing as little as 14,000 cells/mm^3 being adequate. A 25 ml siliconed Erlenmeyer flask with a rubber stopper containing two 21 gage needles was used. Gas was admitted through one needle and escaped through the other; at the end of the gassing procedure, 1 needle was plugged.

Cell densities were maintained at a constant level. This was controlled by a cell count before final dilution. Lactate production varied inversely with the cell density. Cell count variations had little effect when held within the range of 15,000–30,000/mm^3.

Lactic Acid Analysis

This was carried out by the method of Barker and Summerson (1), as described by Hawk *et al.* (3).

Material.—1) Glassware: All the glassware was cleaned and stored in a manner to avoid contamination by hand or animal debris.

2) For protein precipitation: *a)* 0.5 ml cell suspension; *b)* 4.0 ml N/70 sulfuric acid; *c)* 0.5 ml of 3% sodium tungstate.

3) Reagents: *a)* 20% copper sulfate solution; *b)* 4% copper sulfate solution; *c)* calcium hydroxide powdered, U.S.P.; *d)* sulfuric acid, concentrated (low nitrate content (5)); *e)* *p*-hydroxydiphenyl reagent (1.5 mg in 10 ml of 5% sodium hydroxide diluted to 100 ml after solution); *f)* standard lactate solution, prepared by dissolving 0.213 g pure dry lithium lactate or 0.331 g zinc lactate in 100 ml water, adding 1 ml concentrated sulfuric acid and diluting to 1,000 ml; this solution is further diluted, 5 ml of stock to 100 ml solution giving 0.01 mg of lactic acid per ml; 3 standards (5 μg, 10 μg, and 20 μg) were prepared daily.

Procedure.—1) Transfer 2 ml of filtrate to a 15 ml graduated centrifuge tube, add 1 ml of 20% copper sulfate and dilute the solution to 10 ml; add 1 g powdered calcium hydroxide, place Parafilm over the top of the tube, and shake the tube vigorously to obtain complete mixing. During the following 30 min, shake the tube twice; remove the powder by centrifugation; remove the supernatant solution with a pipet to avoid the floating powder.

2) Transfer 1 ml samples of the clear supernatant to tubes, add 0.5 ml of 4% copper sulfate, then 6 ml of concentrated sulfuric acid from a buret drop by drop; boil for 5 min and cool in running water.

3) Add 0.1 ml of *p*-hydroxydiphenyl drop by drop, avoiding the sides of the tubes, shake with a swirling motion, and let stand for 30 min, with occasional shaking.

4) Heat tubes exactly 90 sec in vigorously boiling water and remove to cool.

6) Transfer to colorimeter tubes and read at 560 mμ.

Glucose Utilization

This can be studied on leukocyte suspensions of 14,000 ml[3]. A modification of the method of Folin and Wu (2) and Nelson's (4) modification of the Somogyi method, as described by Hawk *et al.* (3), were used.

Material.—1) For protein precipitation: *a)* 0.01N sulfuric acid; *b)* 7% copper sulfate; *c)* 10% sodium tungstate.

2) Reagents: *a)* Copper reagent, prepared daily by mixing 4 ml of solution B with 96 ml of solution A; solution B contains 150 g of $CuSO_4 \cdot 5H_2O$ in 1,000 ml water with 0.5 ml concentrated sulfuric acid; solution A contains 50 g anhydrous sodium carbonate, 50 g potassium sodium tartrate, 40 g sodium bicarbonate, and 400 g anhydrous sodium sulfate in 1,600 ml distilled water and diluted to 2,000 ml; mixture is filtered and stored at room temperature.

b) Color reagent, prepared by dissolving 100 g ammonium molybdate in 1,800 ml distilled water, adding to this solution 84 ml concentrated sulfuric acid with stirring, and 100 ml water containing 12 g disodium orthoarsenate. The final solution is incubated at 37° C for 2 days and stored in a dark bottle. *c*) Standard glucose solutions, containing 0.025, 0.050, and 0.100 mg/ml prepared from anhydrous glucose in a solution of 0.2% benzoic acid.

Procedure.—1) Deproteinize by mixing 0.5 ml of leukocyte preparation with 5 ml of 0.01N sulfuric acid and adding 0.2 ml of 7% copper sulfate and 0.2 ml of 10% sodium tungstate.

2) Add 0.5 ml of the filtrate to a 10 ml calibrated tube, then 1 ml copper reagent and mix; stopper the tube with a marble, boil for 20 min, and cool in water at room temperature for 1 min; add 1 ml color reagent, mix, and dilute to the 10 ml mark with distilled water.

3) Transfer the total content to colorimeter tubes and read at 540 mμ against a blank prepared with 0.5 ml water.

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D. LYSOZYME TECHNIC FOR MEASURING CELL DAMAGE

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Damage to cells is accompanied by the release of certain cellular contents, including enzymes, into the surrounding medium (1, 2, 6). This phenomenon can be utilized in measuring the degree of injurious or protective effect exerted on cells under defined conditions (2–4).

PROCEDURE

1) Human leukocytes obtained by dextran sedimentation (p. 135) are resuspended in saline to a count of 11,000/mm^3.

* Difco Laboratories, Detroit, Mich

2) Bacto-lysozyme substrate,* 4% in citrate-phosphate buffer, pH 6.2 (5), is subjected to vibration in a 9 KC Raytheon oscillator for 10 min. This stable stock suspension is diluted 5.5 ml to 100 ml with buffer before use.

3) 1.0 ml leukocyte suspension is mixed rapidly with 1.5 ml lysozyme substrate, and an immediate reading is obtained at 540 mμ in a Coleman Junior spectrophotometer (75 × 12 mm), 100% transmission being set by use of a blank tube containing 1.0 ml leukocytes and 1.5 ml buffer. All tests are run in duplicate. After exactly 20 min in a 37° C water bath without agitation, a second reading is obtained.

4) Results are expressed in terms of per cent lysis of lysozyme substrate, calculated by the formula

$$100 - \frac{OD_{20} \times 100}{OD_0}$$

where OD_0 and OD_{20} represent the optical densities at zero time and at 20 min, respectively. An increased lysozyme titer on *in vitro* exposure of leukocytes to various agents (as compared to the titer observed in a sample of the same leukocyte suspension not so exposed but otherwise identically handled) is interpreted as evidence of at least one type of leukocyte damage (1, 2). Similarly, a decreased lysozyme titer on *in vitro* exposure of leukocytes to agents is interpreted as evidence of protection of the cells from injury (3).

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METHODS FOR BIOCHEMICAL STUDY OF SEPARATED HUMAN LEUKOCYTES

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I. GENERAL CELL PREPARATION

The problem of obtaining relatively pure cultures of white cells has been approached from the standpoint of induced peritoneal exudates (15); the use of red cell lysins, such as combinations of gramicidin and lypolecithin (17); flotation on gum acacia (19) or serum albumin (24), taking advantage of the differential specific gravity of erythrocytes and leukocytes; and selective erythrocyte sedimentation employing fibrinogen (11), dextran (21), and phytohemagglutinin (10). The latter methods have proved quite satisfactory (18).

Fibrinogen method.—A 6% solution of sigma bovine fibrinogen (Fraction I) is made up in isotonic saline; 2 parts of this solution to 1 part of blood gives rapid sedimentation, satisfactory white cell yields and a small amount of erythrocyte contamination. Satisfactory sedimentation is not achieved in the cold. No anticoagulant is required, as Fraction I contains approximately 35% citrate by weight.

Dextran method.—Dextran (Type H-1),* average molecular weight 139,000, is made up as a 3% solution in isotonic saline; 2 parts of this solution to 1 part heparinized blood gives fairly satisfactory sedimentation, white cell yield, and contamination characteristics.

Fibrinogen–phytohemagglutinin method.—Further purity of white cell yield can be achieved if 4 mg Bacto-phytohemagglutinin (0528)in† 0.4 ml buffer or saline are added to each 10 ml of white cell rich supernatant from the fibrinogen method. The suspension is then centrifuged at 1,000 rpm for approximately 15 sec, the erythrocyte plug gently loosened with a spatula, the tube gently inverted 10 times, and then recentrifuged momentarily at 500 rpm.

After removal of white cell and platelet rich supernatant, the latter is centrifuged for 3 min at 800 rpm, the plasma containing most of the platelets removed, and the cells resuspended in saline. Centrifugation and washing are repeated, and the final leukocyte suspension is quantitatively enumerated by standard counts in quadruplicate. The original differential formula of the blood

* R. K. Laros Co., Bethlehem, Pa.
† Difco Laboratories, Detroit, Mich.

studied is determined by differential leukocyte counts on peripheral blood.

Analyses for biochemical constituents or enzymic activities are carried out on a suspension of leukocytes whose numbers are accurately known. In certain determinations, the leukocyte suspension may be subjected to mechanical disruption by homogenization, or intracellular constituents may be released by exposure to surface-acting agents, such as saponin, and or to osmotic forces, such as by suspension in distilled water. In some instances, satisfactory analysis is possible only when both mechanical and osmotic forces are utilized. The intact cells may also be employed in studies of certain enzyme systems, such as those operative in glycolysis and respiration.

Results are expressed in many ways: in terms of wet weight, dry weight, cell numbers, cell nitrogen, etc. There are fundamental objections to any mode of expression. However, since both the total number and the morphologic types of leukocytes in the analytic systems are known, these cells are particularly adaptable for expression of data in terms of unit numbers of cells whose composition is known with reasonable accuracy. It must be recognized, however, that certain apparent differences in the biochemical constitution of leukocyte populations may at times be due entirely to different protoplasmic masses in two systems containing an equal number of cells, but of different types. In some instances, therefore, expression of data in terms of cell mass or cell nitrogen is of distinct value. The individual circumstances of the experiment determine the most useful mode of expression of data and whether data should be compared in terms of both cell numbers and some expression of protoplasmic mass. In most circumstances reference to cell numbers suffices.

II. DETERMINATION OF ALKALINE AND ACID PHOSPHATASE ACTIVITY (22)

PROCEDURE

1) Prepare leukocyte suspension as in I. *2)* Prepare incubation mixture as follows:

Reagent	Amount	Final Concentration
Sodium β-glycerophosphate* (0.026M)	9.0 ml	0.02M
Saponin (2% in saline)	0.5 ml	100 mg/100 cc
$MgCl_2$ (0.050M)	0.2 ml	0.001M
Leukocyte suspension	0.3 ml	
Final volume	10.0 ml	

* For alkaline phosphatase, buffered with NaOH and sodium diethylbarbiturate to pH 9.2; for acid phosphatase, buffered with acetic acid and sodium diethylbarbiturate to pH 5.0.

3) Incubate 60 min at 37° C; agitation is unnecessary. In most instances other than leukemia, cell suspensions of 25,000–50,000/mm^3 are adequate. In leukemia, low values often make higher suspension counts practical. Duplicate unknowns are run.

4) Terminate reaction with 2 ml of 30% trichloroacetic acid and remove precipitate by filtration.

5) Sample blank consists of mixture to which the trichloroacetic acid is added prior to addition of cells and reagent blank contains 0.3 ml saline in place of cell suspension.

6) Determine phosphorus by method of Fiske and SubbaRow (4) on 3 ml aliquots of filtrate, adding 0.5 ml molybdate reagent, 0.5 ml aminonaphthol sulfonic acid reagent, and 8.0 ml water.

7) Read blue color after 10 min in Beckman Model B spectrophotometer at 660 mμ. Standard is a reagent blank containing 100 μg of phosphorus.

8) Results are expressed as mg phosphorus liberated from substrate per hour by 10^{10} leukocytes at 37° C.

III. GLUTATHIONE (GSH) CONTENT (8)

PROCEDURE

1) A modification of the nitroprusside method is satisfactory, and neither ascorbic acid nor ergothioneine interfere.

2) Prepare leukocyte rich suspension as in I, centrifuge, resuspend in known volume of distilled water and homogenize for 4 min, using tight-fitting lucite homogenizer (1). Without homogenization, there is incomplete GSH release and falsely low values are found. It is wise to add a small amount of the chelating agent Sequestrene (disodium ethylenediaminetetraacetate) to the original blood–fibrinogen suspension as heavy metal ions catalyze the oxidation of GSH. After initial sedimentation, subsequent manipulations are done in cold at 3–5° C.

3) Deproteinize white cell homogenate aliquots with 2.0 ml of 6% HPO_3 in saturated NaCl containing 3×10^{-5}M Sequestrene. Duplicate aliquots so treated are supersaturated with NaCl, centrifuged 20 min at 3,000 rpm, and filtered through No. 50 Whatman filter paper. 2.0 ml aliquots of filtrates are added to 6.0 ml of a saturated solution of NaCl in ice bath. Final color development is obtained using the nitroprusside method as described by Grunert and Phillips (7). Cuvets *must be* kept in ice water bath till read, since the developed color fades with increasing temperature. Blanks contain 2.0 ml of 2% HPO_3 in saturated saline instead of cell filtrate. Standards contain 100 μg GSH prepared from a stock solution of 2% HPO_3 in saturated NaCl solution containing 20

mg GSH per 100 ml and stored at −17° C without noticeable oxidation over a 3 month period.

4) Final reading is made in Beckman Model B spectrophotometer at 520 mμ wave length.

5) GSH content is expressed as mg GSH /10^{10} leukocytes. Since GSH is also present in erythrocytes and some erythrocyte contamination is present, it is necessary to know the erythrocyte concentration of the leukocyte rich suspension and concomitantly to run a pure red cell GSH determination, using known numbers of red cells lysed in distilled water. The calculated contribution of erythrocytes in the leukocyte rich suspension is then subtracted to obtain the true leukocytic GSH value.

IV. GLYCOGEN CONTENT (23)

PROCEDURE

1) Collect blood as detailed in I. After satisfactory sedimentation, remove leukocyte rich plasma supernatant, and determine leukocyte concentration after mixing by routine counts. Since myeloid leukocytes are the only significant source of blood glycogen (25), this material can be processed directly.

2) Pipet 1 ml of this leukocyte–platelet suspension into triplicate boiling tubes containing NaOH in a final concentration of 30%. Centrifuge remainder of suspension cell free and put 1 ml of cell-free fluid in each of 2 boiling tubes and process in the same way as the cell-containing material. These serve as the basis for the blank and for the standard (after later addition of a known amount of glucose).

3) Cover tubes with glass marbles, digest by boiling 45 min, and add to each tube sufficient 95% alcohol to bring final alcohol concentration to 60%. Glycogen precipitates; to attain maximum yield let stand in cold, centrifuge, and wash precipitate twice with 60% alcohol.

4) Dry in oven at approximately 100° C. Suspend the dried material of unknowns and blank in 4 ml distilled water. To the standard tube add 4 ml distilled water containing 50 μg glucose. Add 8 ml anthrone reagent (12, 16) (0.2% anthrone in concentrated H_2SO_4). Read after 10 min at 620 mμ in Beckman Model B spectrophotometer.

5) Precautions: Prepare anthrone reagent fresh, use sulfuric acid of high purity, avoid contamination with lint or other material reacting with this reagent, and add anthrone reagent at uniform speed to obtain the good mixing and heat elaboration upon which satisfactory and uniform color development depend. Some prefer

to control the temperature of reaction by adding anthrone to tubes in a boiling water bath.

CALCULATION

$$\mu g \text{ glycogen in 1 ml aliquot} = \frac{50 \times V}{1.11 \times S}$$

where V = optical density of unknown; S = optical density of 50 μg glucose standard; 1.11 = factor of conversion of glucose to glycogen. Knowing the number of cells in the 1 ml aliquot and the differential formula of subject's blood, glycogen can be readily expressed in terms of mg/10^{10} total leukocytes, or, perhaps more significantly, per 10^{10} myeloid leukocytes.

V. GLUCURONIC ACID CONTENT (6)*

PROCEDURE

1) Prepare and wash cells as in I. After obtaining suspension count, centrifuge cells, resuspended to exactly known volume in distilled water, and homogenize for 5 min, using tight fitting lucite homogenizer. Unless both osmotic and mechanical forces are applied, poor or incomplete release of glucuronic acid occurs. Erythrocytes contain no demonstrable glucuronic acid.

2) Add 2.0 ml mixed homogenate to 6.0 ml distilled water and mix. Add 1.0 ml each of 10% sodium tungstate and 0.66N sulfuric acid. The volume relationships are of great importance if adequate deproteinization is to be obtained. Filter after 10 min through No. 40 Whatman paper. Add 3.0 ml aliquots of filtrate to duplicate glass-stoppered NPN tubes containing 2.0 ml 0.2% naphthoresorcinol in distilled H_2O and 2.0 ml concentrated HCl. Blank contains 3.0 ml distilled H_2O and standard 20 μg of glucuronic acid in 3.0 ml distilled H_2O in place of filtrate.

3) Place in boiling water bath for 60 min, then immerse in cold water (5° C) for 10 min and add 5.0 ml 95% alcohol to dissolve blue pigment. After agitating tubes, add 10.5 ml water-saturated toluene and extract vigorously for $1^1/_2$ min. Centrifuge at 2,500 rpm to eliminate emulsification, transfer toluene extract to cuvets and read at 565 mμ, using reagent blank without cells as reference.

4) Results may be expressed as μg/10^{10} leukocytes.

VI. ESTERASE AND LIPASE ACTIVITY (9)†

PROCEDURE

1) β-Naphthol acetate is used as substrate for esterase activity and β-naphthol laurate for lipase activity. These are prepared ac-

* The determination is a modification of Fishman's (3).
† Modified from the method of Nachlas and Seligman (13) for serum.

cording to the description of Nachlas and Seligman (13, 14). The former is stored as a stock solution of 500 mg reagent in 100 ml acetone in the refrigerator; the latter is similarly stored as a solution of 50 mg reagent in 25 ml acetone.

2) Esterase substrate is prepared by adding 2.0 ml of stock solution to 70.0 ml tris buffer (tris(hydroxymethyl)aminomethane at pH 7.4 and in 0.05M concentration) and 8.0 ml water *immediately before use*. Lipase substrate is prepared by adding 1.0 ml stock solution with a submerged pipet while swirling into 19.0 ml tris buffer (pH 7.4 and in 0.05M concentration) immediately before use.

3) Separated, washed leukocytes are prepared as saline suspension as detailed in I.

4) The esterase incubation is carried out as follows: Pipet into NPN tube 1.0 ml cell suspension and 2.0 ml glass distilled water. Let stand for 5 min. Add 4.0 ml substrate. Incubate 45 min at 4° C (higher temperature may be used, but the marked esterase activity is conveniently measured at 4° C). Add 1.0 ml of freshly prepared solution of naphthanil diazo blue B (80 mg tetrazotized diorthoanisidine* in 20 ml cold water); 3 min later add 1.0 ml of 40% trichloroacetic acid and extract color immediately with 15 ml ethyl acetate. Blank is prepared by adding cells after addition of trichloroacetic acid. Color intensity of duplicate unknowns is measured at 540 mμ and related directly to a standard of β-naphthol.

5) Lipase activity is similarly determined except that the initial incubation mixture contains 1.0 ml cell suspension, 1 ml of 8×10^{-2}M sodium taurocholate solution as an esterase inhibitor (14, 4), 5 ml of β-naphthol laurate as substrate, and the incubation period is 5 hr at 37°.

6) Values may be expressed as mg of β-naphthol liberated per hour from substrate by 10^{10} cells at 4° C in case of esterase, and at 37° in case of lipase.

7) Erythrocytes contain significant esterase activity but no demonstrable lipase activity under these conditions. The erythrocyte contamination of white cell suspensions must therefore be accurately known in the esterase determination, and a concomitant determination of pure erythrocyte esterase must be done in order to make the appropriate correction and obtain a true leukocyte esterase value.

* Dajac Labs.

VII. β-GLUCURONIDASE CONTENT (5)

PROCEDURE

1) Prepare leukocyte suspensions of 5,000–15,000 cells/mm^3 as in I.

2) Assay β-glucuronidase by a modification of the method of Fishman and co-workers (2, 20), using phenolphthalein-mono-β-glucuronide as substrate. The incubation mixture is 0.1 ml acetate buffer, 0.1M, pH 4.5; 0.1 ml phenolphthalein-mono-β-glucuronide (3), 0.01M; 0.4 ml saponin, 0.5%; 0.5 ml leukocytes in saline; final volume, 2.0 ml. Incubate 3 hr at 38° C in water bath and stop reaction by adding 1.0 ml 10% trichloroacetic acid. Unknowns are run in duplicate.

3) Prepare suitable blank and standard tubes; in the case of the blank, cells are added after addition of trichloroacetic acid; and in the case of the standard, phenolphthalein-mono-β-glucuronide is added after trichloroacetic acid to a reaction mixture containing 40 μg phenolphthalein.

4) The liberated phenolphthalein is measured as recommended by Fishman *et al.* (2), the final color development being measured at 540 mμ wave length in the Beckman Model B spectrophotometer using large Coleman cuvets and a final volume of 10 ml.

5) Activity may be expressed as mg phenolphthalein liberated per hour by 10^{10} leukocytes at 38° C.

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PEPTIDASES OF HUMAN LEUKOCYTES

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HUMAN LEUKOCYTES have been shown to contain at least three powerful peptidases (2, 3). One dipeptidase is rather specific for glycylglycine (GG) and requires cobalt as the activating ion. Its optimal activity in phosphate buffer is at pH 7.7 in the presence of 10^{-4}M Co^{++}. Another dipeptidase requires zinc ion. Its substrate specificity appears to be rather broad, since a group of dipeptides have been found to be hydrolyzed under very similar conditions. The presence of Ca^{++} has been noted to retard the inactivation of the enzyme. Glycyl-L-leucine (GL) is split at a faster rate than most other dipeptides tested; optimal activity was found at pH 7.8 in the presence of 10^{-4}M Zn^{++} and 10^{-3}M Ca^{++}. The leukocytic tripeptidase, like the enzyme in serum and erythrocytes, does not require the addition of an activating ion, though it undoubtedly too is a metal protein (2). Glycylglycylglycine (GGG), while not its best substrate, is the one most readily available. Only one bond is hydrolyzed, and the optimal activity is at pH 7.0. In leukocytes from 25 normal persons the mean activities and standard deviations were found to be: GG, 1.1 ± 0.3 μM/hr/10^6 cells; GL, 7.2 ± 1.6 μM/hr/10^6 cells; GGG, 4.5 ± 1.1 μM/hr/10^6 cells.

The increase of free amino groups during the enzymic peptide hydrolysis is measured by means of the colorimetric ninhydrin method of Moore and Stein (4), which was applied to the analysis of serum peptidases for the first time by Schwartz and Engel (5).

PROCEDURE

Apparatus.—1) Constant temperature bath set at 38° C. *2)* Glass-stoppered Erlenmeyer flasks, 10 or 25 ml capacity. *3)* Pipets, 0.2, 0.5, 1, 2, 5, 10 and 50 ml volumetric; 1.0 ml graduate. *4)* Matched colorimeter tubes for use with Coleman junior spectrophotometer, model 6-A. Pyrex culture tubes #9820, 18 × 150 mm, can be used; they are matched by determining the optical density of a methyl red solution of suitable concentration in 0.03N HCl at 525 mμ; those tubes which give readings within 0.010 unit are selected. *5)* Aluminum caps for colorimeter tubes.

Reagents.—1) M/12 phosphate buffers (pH 7.7 for GG; pH 7.8 for GL; pH 7.0 for GGG). *2)* 0.001M $CoCl_2$. *3)* 0.001M $ZnSO_4$ + 0.01M $CaCl_2$. *4)* 0.050M GG, GL, GGG. *5)* pH Adjusting solutions for peptides, 0.016N NaOH for GG; 0.019N NaOH for GL; 0.004N NaOH for GGG. *6)* 0.025M peptides, adjusted to the pH

of the buffers. Mix equal volumes of 0.050M peptides with the corresponding adjusting solutions. *7)* Standard solution of 0.001M L-leucine. *8)* 1% picric acid. *9)* 0.2M citrate buffer, pH 5.0. Dissolve 21.008 g citric acid $C_6H_8O_7 \cdot H_2O$ (reagent grade) in 200 ml N NaOH and dilute to 500 ml. The pH of the buffer when diluted with an equal volume of water should be 5.0 ± 0.1. *10)* Ninhydrin reagent. A fresh solution should be prepared daily, as needed. For instance, 2.600 g ninhydrin* are dissolved in 65 ml 2-methoxyethanol.† To this solution add 65 ml of a solution of 104 mg $SnCl_2 \cdot 2H_2O$ in citrate buffer, pH 5.0. *11)* Diluent solution, containing equal volumes of *n*-propyl alcohol‡ and water.

Preparation of White Cells

In our laboratory dextran rather than beef fibrinogen is now used to accelerate the agglutination of erythrocytes, since it gives cleaner preparations of white cells and is easier to handle; it has the additional advantage that platelets may be separated from the remaining plasma. The anticoagulant is Sequestrene (disodium ethylenediamine tetra-acetate). The following solution is made up: 8.35 ml of 18% dextran,§ 20.0 ml of 1.5% Sequestrene in 0.9% NaCl, and 21.65 ml of 0.9% NaCl. 1 part of this solution is mixed with 2 parts of blood immediately after venipuncture and allowed to settle. The plasma containing leukocytes and platelets is centrifuged at 800 rpm (about 95*g*) and 40° C for 30 min.

To preserve the viability of the leukocytes, silicone-treated glassware is used, and 4% serum albumin, human or beef, in 0.9% saline solution serves as the medium in which to suspend the cells. (The presence of albumin or another protective colloid is absolutely essential to preserve enzyme activities during peptide hydrolysis.) The cells are washed twice with this solution, followed by centrifugation at 1,500 rpm (400*g*) for 10 min. The final suspensions, which preferably are adjusted to contain 25,000 cells/mm³, are frozen before the enzyme activities are determined; this helps to rupture the cell membranes. The preparations should not be stored at −20° C for more than a day or two, since the dipeptidases are not very stable even at that temperature.

Immediately before the enzyme test, 4 vol of water are added to the frozen cells. This mixture is used for GG; an additional 5-fold dilution is made with 0.8% albumin for GL and GGG.

* Nutritional Biochemicals Corporation, Cleveland, Ohio.
† Eastman P 2381.
‡ Eastman 848.
§ Pharmacia, Uppsala, Sweden.

Enzyme Reactions

For each leukocyte preparation, 3 flasks are set up with the following solutions:

1) 1.0 ml M/12 phosphate buffer, pH 7.7, 0.25 ml H_2O, 0.50 ml leukocytes 1:5, 0.25 ml 0.001M $CoCl_2$, and 0.50 ml 0.025M GG, pH 7.7.

2) 1.0 ml M/12 phosphate buffer, pH 7.8, 0.25 ml H_2O, 0.50 ml leukocytes 1:25, 0.25 ml 0.001M $ZnSO_4$ + 0.01M $CaCl_2$, and 0.50 ml 0.025M GL, pH 7.8.

3) 1.0 ml M/12 phosphate buffer, pH 7.0, 0.50 ml H_2O, 0.50 ml leukocytes 1:25, and 0.50 ml 0.025M GGG, pH 7.0.

Immediately after adding the peptides, the contents of the flasks are mixed well; 0.5 ml samples are then withdrawn and added to 2 ml portions of picric acid contained in centrifuge tubes. (When a number of white cell preparations are tested, it is usually sufficient to sample only one set at zero time, since the cells at the dilutions used do not contribute within the error of analysis to the ninhydrin color.) The flasks are incubated at 38° C; after 2 and 4 hr additional samples are withdrawn and precipitated in a like manner. All tubes are then centrifuged at 2,200 rpm for 10 min and the supernatant solutions are decanted for the ninhydrin assay.

To avoid contamination with traces of ammonia from the air, all solutions should be kept covered whenever possible.

Colorimetric Ninhydrin Method

Determinations are preferably done in triplicate. Pipet 0.2 ml portions of the various samples into a series of colorimeter tubes. Also included are tubes containing 0.2 ml portions of water for the reagent blank and of leucine standard. All tubes receive 1 ml of ninhydrin reagent. With the caps put in place, immerse the tubes in boiling water for 20 min, then cool in a bath with cold water. Next add 10 ml of the 1:1 propanol-water diluent, mix the contents of the tubes well, and determine the optical densities at 570 mμ. The reagent blank is read against a reference tube containing the diluent alone; the other tubes are read against the reagent blank.

In the simplified method, in which a table is used (Table 9), the 2 hr and 4 hr samples are read against the corresponding zero time samples and the differences in optical densities are obtained directly.

CALCULATION

As was shown by Moore and Stein (4) for the ninhydrin test, Beer's law does not hold for optical densities above 0.5 under the

conditions applied. The concentration of color-producing substances was nevertheless chosen to utilize the entire colorimeter range, in order to increase the accuracy of the test. The necessary corrections can be calculated with close approximation by the use of an empiric formula:

$$\text{Calculated density} = 1.12 \times \text{observed density} - 0.075$$

Only readings above 0.625 are to be corrected.

To obtain the fraction p of a peptide which has been hydrolyzed by an enzyme during the time of observation, the difference of corrected optical densities between the final and the initial sample is multiplied by a conversion factor. The factors differ for different peptides, since the color yields of various peptides and amino acids are not the same. Each conversion factor is best determined in the following way. Peptide in 0.001M solution is mixed in several proportions with a solution containing the 2 reaction products in 0.001M concentration, and the ninhydrin color is determined in the usual way. When the corrected densities are plotted against the concentrations, in mM/liter, of either reaction product, the reciprocal of the slope of the straight line is the conversion factor. The conversion factors for the 3 peptides of this study, based on an optical density of 0.530 for 0.001M leucine, are: GG, 1.67; GL, 1.90; and GGG, 1.72. At higher values for the leucine standard, the factors are correspondingly smaller, and vice versa.

To translate accurately the fraction p of a peptide, which has been hydrolyzed, into enzyme activity, the kinetics of the reaction must be known. For this purpose, the whole reaction curves of the 3 leukocytic enzymes have been studied carefully and the results have been evaluated by the method of "curve fitting of enzymatic reactions based on the Michaelis-Menten equation" (1). This method has yielded the following parameters: GG, $r' = 0.97$; GL, $r' = 1.55$; GGG, $r' = 2.35$.

When corrected with the help of these parameters, the fractions p become the values f, which represent points on the tangent of the reaction curve at zero time; the slope of this tangent, therefore, indicates the initial rate of the reaction. After multiplication with the initial peptide concentration in μM/ml ($=5$) and divided by the time in hours and by the number of cells in millions/ml of reaction mixture, the initial activities are obtained in μM peptide hydrolyzed/hr/10^6 cells.

For simplification, a table has been constructed which allows one to obtain activities directly from the observed differences in optical densities for a reaction time of 2 hr and a leukocyte count of 25,000 cells/mm^3 in the frozen preparation (Table 9). For other

TABLE 9.—Peptidase Activities of Leukocytes

($M/hr/10^6$ cells)

Standard leucine, D = 0.530; reaction time, 2 hr; other conditions in text

Optical Density Increase	GG D = .550	GL D = .570	GGG D = .500	Optical Density Increase	GG D = .550	GL D = .570	GGG D = .500
0.005	0.02	0.12	0.10	0.255	1.35	7.73	6.42
.010	0.04	0.24	0.21	.260	1.38	7.92	6.57
.015	0.06	0.36	0.32	.265	1.42	8.12	6.72
.020	0.08	0.49	0.42	.270	1.45	8.31	6.88
.025	0.11	0.62	0.53	.275	1.49	8.51	7.04
0.030	0.13	0.75	0.64	0.280	1.52	8.72	7.20
.035	0.15	0.88	0.75	.285	1.56	8.93	7.36
.040	0.17	1.01	0.87	.290	1.60	9.14	7.53
.045	0.19	1.13	0.98	.295	1.63	9.35	7.69
.050	0.22	1.26	1.10	.300	1.67	9.57	7.86
0.055	0.24	1.39	1.20	0.305	1.71	9.80	8.02
.060	0.26	1.52	1.32	.310	1.75	10.02	8.20
.065	0.29	1.65	1.43	.315	1.79	10.25	8.37
.070	0.31	1.79	1.55	.320	1.83	10.49	8.55
.075	0.33	1.92	1.67	.325	1.87	10.73	8.73
0.080	0.36	2.06	1.79	0.330	1.91	10.98	8.91
.085	0.38	2.20	1.91	.335	1.95	11.24	9.09
.090	0.41	2.34	2.03	.340	1.99	11.50	9.27
.095	0.43	2.48	2.15	.345	2.03	11.77	9.45
.100	0.46	2.62	2.27	.350	2.07	12.05	9.63
0.105	0.48	2.76	2.39	0.355	2.12	12.33	9.82
.110	0.51	2.91	2.51	.360	2.17	12.62	10.01
.115	0.53	3.05	2.63	.365	2.21	12.92	10.20
.120	0.56	3.19	2.75	.370	2.26	13.22	10.39
.125	0.58	3.33	2.87	.375	2.31	13.53	10.59
0.130	0.61	3.48	3.00	0.380	2.36	13.85	10.79
.135	0.63	3.63	3.12	.385	2.41	14.19	10.99
.140	0.66	3.79	3.25	.390	2.46	14.55	11.19
.145	0.69	3.95	3.38	.395	2.51	14.93	11.40
.150	0.71	4.11	3.51	.400	2.57	15.33	11.61
0.155	0.74	4.26	3.63	0.405	2.63	15.75	11.83
.160	0.77	4.42	3.76	.410	2.69	16.19	12.06
.165	0.80	4.57	3.89	.415	2.75	16.65	12.30
.170	0.83	4.73	4.02	.420	2.81	17.14	12.55
.175	0.86	4.89	4.15	.425	2.87	17.67	12.81
0.180	0.89	5.06	4.29	0.430	2.94	18.26	13.08
.185	0.91	5.22	4.42	.435	3.01	18.91	13.36
.190	0.94	5.38	4.56	.440	3.08	19.63	13.65
.195	0.97	5.54	4.70	.445	3.16	20.43	13.95
.200	1.00	5.72	4.83	.450	3.24	21.32	14.27
0.205	1.03	5.91	4.96	0.455	3.32		14.61
.210	1.06	6.09	5.10	.460	3.41		14.97
.215	1.09	6.27	5.25	.465	3.50		15.35
.220	1.13	6.45	5.40	.470	3.60		15.75
.225	1.16	6.63	5.54	.475	3.70		16.16
.230	1.19	6.81	5.69	.480	3.81		16.59
.235	1.22	6.99	5.83	.485	3.92		17.05
.240	1.25	7.17	5.97	.490	4.04		17.55
.245	1.28	7.35	6.12	.495	4.18		18.10
.250	1.32	7.54	6.27	.500	4.34		18.76

cell counts, corrections are to be applied; for 4 hr samples, the figures are divided by 2. The figures in the table are based on the density readings for leucine and for the peptides (zero time samples) as indicated. Corrections for small deviations from these values are usually not required, especially if one considers that the accuracy of cell counting is not very great. Activities obtained from the 2 hr and 4 hr samples usually agree within less than 5%.

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METHODS DEVELOPED FOR CULTURE OF HUMAN LEUKOCYTES

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THREE SIMPLE METHODS of culture of human leukocytes are presented. Maintenance of continuous growth with two of the methods depends on principles developed with the gradient culture method (4). All three methods are adaptable to the study of any type of cell, and to the investigation of many facets of growth *in vitro*.

The first, or *gradient culture* method, provides a continuous gradient in cell numbers, oxygen tension, oxidation-reduction potential and cell conditioning factors, and makes possible continuous removal of the most mature stages. It is best adapted for an initial study of a particular cell type to determine the conditions which ideally should be duplicated for all cells in the other cultures.

The second, or *bulk culture* method, is designed to provide large quantities of cells for chemical, tracer and metabolic studies. It is best adapted also for long term culture of cells. By this method two strains of cells isolated from the blood of patients with acute monocytic leukemia are continuously multiplying after 8 mo. Several strains of cells isolated from bloods of patients with chronic granulocytic leukemia have been maintained for 3 mo, and one strain is still increasing in numbers after 100 days to this date. Morphologically, the cells isolated from chronic granulocytic leukemias resemble immature cells of the granulocytic series, and give typical peroxidase staining reactions.

The third, or *inverted slide cap* method, was developed to study growth quantitatively in the presence of chemotherapeutic agents or other variables, in controlled replicate cultures. It also preserves the feature of the gradient culture method of permitting examination of the cells in the position in which the growth had taken place. Either phase contrast microscopy, or fixed and stained preparations of the cells can be used. With this method we have been able to carry cells of granulocytic series isolated from human leukemic bloods through 6 transplants with continued growth over a period of 150 days to date.

Preparation of Materials

The materials used for each method are described in the method to which they apply. Certain equipment is used in all methods, such as, 1, 10, 20, 30, and 50 ml syringes; 18 and 20 gage needles,

and 6-inch 20 gage needles; 15 and 50 ml centrifuge tubes; and size 14 and 100 vaccine stoppers. The glassware is cleaned by standard tissue culture methods, rubber stoppers are boiled for 1 hour in 10% NaOH and washed overnight in running tap water, rinsed in 20% HCl, washed again in tap and distilled water, and stored in 70% alcohol. As many manipulations as possible are carried out by puncturing through the vaccine stoppers with syringe and needle. The outer surface of the vaccine stoppers is sterilized by allowing a pool of 70% alcohol to stand on the cap for a few moments. The puncture is made through this pool.

Culture Medium

Of the several different culture media used, the following composition has given the most rapid growth of leukocytes: 400 ml pleural fluid (from patients with carcinoma);* 300 ml TC Medium No. 199 (0477);† 300 ml balanced salt solution.‡ To each liter of this medium is added 1.4 ml of a 1% solution of phenol red (phenolsulfonphthalein), and 100,000 units of potassium penicillin G.

Cell Separation

Blood is collected from a suitable donor of cells for culture in sterile vaccine-capped 50 ml centrifuge tubes containing 0.1 ml heparin for each 10 ml blood (0.1 mg heparin/1 ml blood). The leukocytes are separated by centrifugation at 50*g* for a time sufficient to sediment the erythrocytes and leave the leukocytes suspended in the supernatant plasma—about 7–10 min for most bloods. If the sedimentation rate is not greatly elevated, 10–20 mg Bacto-phytohemagglutinin (0528)† should be added before centrifugation (1). The plasma containing the leukocytes is withdrawn in a syringe using a long needle, then transferred to a sterile vaccine-capped 15 ml centrifuge tube. The leukocytes are spun down at 200*g* for 5 min. Too long a time at this step will pack the cells, hence the time of centrifugation must be carefully controlled. The clear cell-free plasma is drawn off and discarded, and the cells are quickly resuspended in balanced salt solution. This washing is repeated twice more, after which the cells are suspended in culture medium. A sample of this suspension is removed and a cell count

* Other protein sources, such as lyophilized human serum (Hyland Laboratories, 4334 Sunset Blvd., Los Angeles, Calif.) have given satisfactory, but not as consistent results. Pleural fluid from patients in congestive failure proved less satisfactory.

† Difco Laboratories, Detroit, Mich.

‡ Composition: NaCl, 7.64 g; KCl, 0.37 g; $CaCl_2$, 0.224 g; $MgCl_2$, 0.20 g; $NaHPO_4$ (anhydrous), 0.12 g; KH_2PO_4, 0.03 g; $MgSO_4 \cdot H_2O$, 0.07 g; CH_3COONa, 0.50 g; dextrose, 4.00 g; H_2O to make 1,000 ml.

is made by leukocyte counting technic. The number of ml necessary to contain the desired inoculum for the culture is computed from this count.

GRADIENT CULTURE METHOD

The objectives of this method are to determine the gradient factors for the various cell types. This information is then applied to the other methods, determining the total initial inoculum for a given depth of medium. For each type of cell there seems to be an optimal depth for a particular initial concentration of cells per unit volume of medium and per unit area of surface on which the cells grow. As both cells/unit volume and total depth influence the gradient, a rough guide to optimum level is the product of the cell count in thousands/mm^3, and depth in cm of the best growth. This product, for any particular cell, we call the "gradient factor" for that cell type. The approximate ranges for the gradient factors of the cell types studied to date are: bronchogenic carcinoma, 0.3–0.5;* plasmocytic series, 0.5–2.5; granulocytic series, 1.5–6.0; monocytic series, 1.0–8.0; lymphocytic series, 2.0–10.0; erythrocytic series 3.5–12.0. These figures give the total range in all cultures; the range in each individual culture is much narrower.

A suspension type of culture is used which results in a continuous gradient of oxygen tension, CO_2 tension, oxidation-reduction potential, and cell numbers. The bottles used in this method are wide-mouth, screw-cap French squares of 1 pt capacity. The slides are sterile standard 3 × 1 inch glass microscope slides. The bottles are sterilized with the caps loosely in place. The slide is introduced and removed with long sterile sponge forceps. The slide rests at about a 45° angle and requires 170 ml of medium to cover it entirely. Enough of the cell suspension is introduced to give the desired number of cells in the culture. The initial inocula have varied from 400 to 4,000/mm^3, but 1,000/mm^3 has been used most frequently. The culture is sampled at 2–10 day intervals, depending on the cell count; the slide is then removed with the sterile forceps and, after thorough mixing, 2 ml of the culture is aspirated. The slide is air dried, and stained by Wright's method. The 2 ml sample is used for cell counts and smears for differential counting. The number of cells on the slide may be calculated by counting the number of cells in a central longitudinal strip the length of the slide with a Miller disk (1) and multiplying by the ratio width of strip:width of slide. The slide is replaced with a sterile slide, necessary pH adjustments are made by adding CO_2 or replacing the gas

* Other carcinoma cells have different gradient factors.

in the bottle, and the culture is returned to the incubator until the next sampling time.

GRADIENT PRINCIPLES APPLIED TO BULK CULTURES

The culture flasks we have used in this method are rectangular, hard glass bottles with nearly flat sides having a total volume of 255 ml. The larger side has an area of 6,000 mm^2, and forms the bottom surface on which the cells adhere. 40 ml of culture medium in these containers gives an average depth of 6.6 mm.

The gas phase above the culture medium is room air to which sufficient CO_2 is added to give a pH of 7.4 (about 4% -7% CO_2). After sterilization in an electric oven, the culture flasks are capped with large sterile vaccine stoppers; the culture medium and cells are introduced with syringe and needle. Initial inocula from which growth has been started have varied from 15-60 million cells, depending on the gradient factor of the cells. After varying periods in culture, much smaller inocula will initiate growth in subsequent subcultures. Subculture or medium change is necessary when the pH falls to near 7.0. The air in the culture flasks is replaced at the same time that the medium is changed. Some cells seem to grow better if only half the medium is changed at one time. Subcultures are necessary when the cells cover nearly all the available space on the glass surface. It is most important that the cultures not be disturbed between times of sampling or subculture, as this temporarily destroys the gradient and interferes with cell growth. A good practice is to run replicate cultures, one of which is examined under low power optics at 1-3 day intervals until the time for subculture is determined. This disturbed culture frequently deteriorates and a second replicate has to be substituted.

GRADIENT PRINCIPLES APPLIED TO SLIDE CAP CULTURES

The culture chambers used in this method (3) were constructed from machine shell vials (Kahn antigen mixing vials) which were ground to a smooth flat lip with emery powder on a plate glass surface. After sterilization, the lip of these vials was dipped in melted beeswax and cooled in the inverted position. When the wax had solidified, a previously prepared culture suspension was introduced into the vials. A convenient amount of culture for the granulocytic or monocytic series is 3.5 ml of a suspension containing 1,000 cells/mm^3, giving a depth of 2 cm in the vial. A sterile glass slide is placed on top of each culture and the vials are placed in the incubator to allow the gas phase to equilibrate to 37.5° C. 3 hr are

allowed for gas equilibration, after which the vials are removed from the incubator, one at a time, and the slide is sealed to the vial by quickly melting the wax with a Bunsen flame directed at the top of the slide. After the slide has cooled, the cultures are inverted and returned to the incubator with the slide forming the bottom of the culture chamber. At the desired intervals, cultures to be sampled are gently agitated, to suspend the cells not attached to the slide, and the slide is removed, leaving the medium in the vial. The slide is air dried and stained, or immersed in a fixative solution, depending on the staining technic to be used. The slide may be covered with a cover glass previously rimmed with petrolatum and examined as a phase contrast mount.

The cells remaining in the supernatant medium in the vial can be counted by transferring a small quantity of the well-mixed suspension to a hemocytometer. The total number in the culture is obtained by adding to this count the number of cells counted on the slide. The remaining supernatant is centrifuged in 5 × 50 mm tubes to make smears for differential counts. If it is desired to make subcultures, this supernatant is divided between 2 waxed vials, fresh medium is added to bring the volume up to 3.5 ml, and covered with a new slide. After equilibration in the incubator, the slides are sealed in place. This is the method of choice for most quantitative studies, once the "gradient factor" has been determined.

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TECHNIC FOR THE STUDY OF LEUKOCYTIC FUNCTIONS IN MAN

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EXUDATIVE CELLS can be studied in consecutive permanent preparations from individual experimental human skin lesions hour by hour, using, with few exceptions, any of the staining procedures suitable for blood studies and presently available types of microscopy.

PROCEDURE

The volar surface of the forearm is the site chosen for the tests in man, although any other convenient region can be employed. The skin is shaved and cleansed with alcohol. The epithelium is scraped away, not cut, from an area 4 mm in diameter, with a sterile scalpel. The proper depth is reached when fine bleeding points indicate that the papillary layer of the corium has been reached. Frank bleeding should be avoided.

Since the trauma of the technic itself excites some inflammation, the time at which the epithelium is removed is the time when the experimental inflammation begins. A solution or suspension of the inflammatory agent to be studied is next applied to the denuded corium with a bacteriologic loop. The lesion is then covered at once with the first of a series of sterile, chemically clean, cover slips which is surmounted in turn by a square of cardboard and by surgical adhesive tape. The tape measures approximately 2 × 2 in. and is so applied that its center overlies the lesion. For convenience in handling, a number of kits containing a cover slip and cardboard square wrapped together in a paper wrapper are prepared at one time. They are oven-sterilized in Petri dishes and can be removed individually as needed.

The cells of the exudate migrate to and flatten themselves upon the under surface of the cover slip in 30 min to 1 hr. The first cover slip is then removed and rapidly air-dried for blood stains (3, 6, 7), appropriately fixed for histochemical study (2, 4) or frozen-dried for electron microscopy (5). At the same time, a second cover slip is placed over the same lesion and the process is repeated as desired at timed intervals.

If the series of cover slips so obtained are to be stained like blood slides, they are mounted, cell side up, on an ordinary glass slide

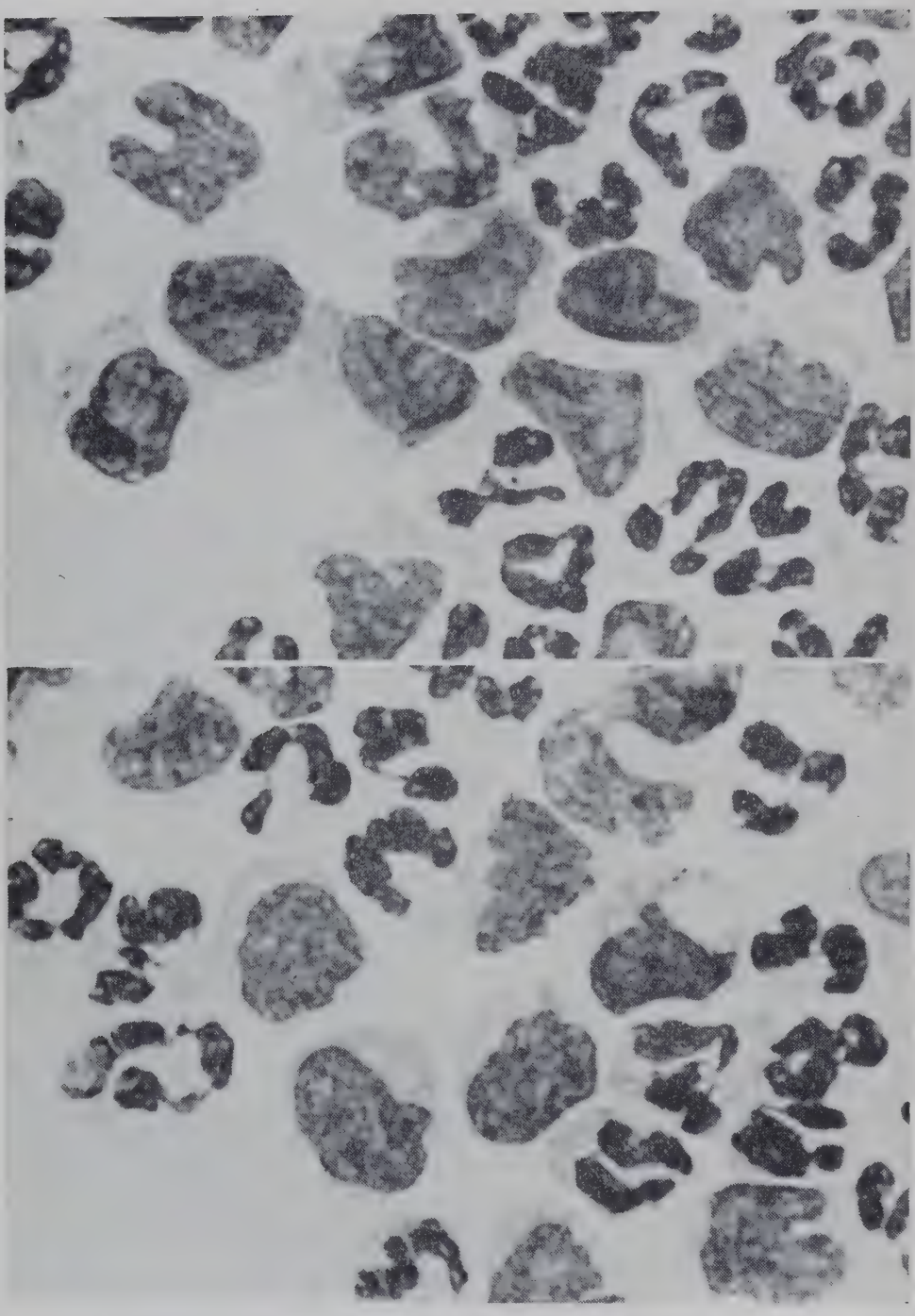

FIG. 1.—Slightly hypertrophied lymphocytes, a monocytoid cell, and 13 shrunken neutrophils in the 12th hour of inflammation in man. 1,400×. AFIP Neg. 93504.

FIG. 2.—Same lesion as in Figure 1; 9 lymphocytes, 3 monocytoid cells and 10 shrunken neutrophils in the 14th hour of inflammation. 1,400×. AFIP Neg. 93505.

by means of a drop of rubber cement. The entire slide can be covered with the staining solutions. We have usually employed May-Grunwald-Giemsa or Wright-Giemsa stains. After staining and drying, the cover slip is mounted permanently, cell side down, on a glass slide. If the series of cover slips are to be studied in the electron microscope, the cover slips are first surmounted by a thin film of Formvar or collodion. The specimen screens are placed over the cell-bearing area under light microscope observation. Direct specimen mounting is obtained by the stripping method described in detail previously (5).

The cover slips exhibit a single layer of exudative cells on their undersurface (Figs. 1 and 2).

The technic described above for man has been modified in the following manner (1) for the rabbit. A tongue depressor is fitted as a splint into the hollow of the inner aspect of the shaved ear. The splint is fixed in place by strips of adhesive tape, one encircling the base of the ear and the other 2 cm from its tip. The outer aspect of the ear then presents a smooth bare area with unimpaired vascular supply. The lesion is then produced and studied, as described above for man.

The *advantages* of the technic are: *1)* It is almost without discomfort to the patient. *2)* Single lesions can be sampled for their cellular content throughout the first 48 hr or more of inflammation in man; in this way, a series of permanent, fixed preparations of *in vivo* samplings of the cellular exudates can be obtained. *3)* Air-dried (3, 6, 7), wet-fixed, or histochemical methods (2, 4) of cell analysis can be studied by light (1–4, 6, 7), phase, or electron microscopy (5) with this technic.

The *disadvantages* are: *1)* The technic is not immediately suitable for supravital staining procedures. *2)* The same cell cannot be followed throughout the course of inflammation, only the cell populations.

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SECTION IV

Methods for Study of the Histology and Cytology of the Retina

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INTRODUCTION

THE STUDY OF the structure of the retina is perhaps one of the more intricate problems which confront the cytologist. The tissue itself is of considerable delicacy and is enclosed within a tough impenetrable fibrous capsule formed by the cornea and sclera, to which it and the choroid membranes are but loosely attached. It is extremely susceptible to postmortem changes and the high lipoid content causes great structural modifications when fat solvents, such as are used in many embedding techniques, are employed.

Because of these and other difficulties it has been considered worth while to present this section on some of the methods available for its study. Moreover, the information provided may also be applicable to many other tissues and, since the study of the retina necessitates a particularly careful consideration of the aims and objects of various histological and cytological procedures and of the results which can be obtained by their use, this, too, may be useful elsewhere.

The architecture of the retina may be studied from many different points of view, and the methods required in each case are correspondingly different.

1) As a first approach, and for purposes of comparative anatomy, it is clearly necessary to study the micro-anatomy and histology of the various cell layers and structures in their natural relationship

to one another. In this task the main necessity is to preserve the salient structures in as nearly as possible the same condition as they were in life. To achieve this end, some sacrifice may have to be made in the detailed cytological features, but obviously the smaller this sacrifice the better.

2) The relation of structure to function necessitates very complete studies of the cellular structures in different parts of the retina, so that the various regional peculiarities of vision may be correlated, or not, with the various structures observed. These studies not only require the best possible analysis of the cytological characteristics, but, in addition, further information may have to be sought at the expense of some of the detail found in the usual histological picture, because many of the cytological techniques are incompatible with the use of rapidly penetrative fixatives, and some studies require the complete isolation of cells or even of parts of cells.

The study of the intercellular and neural connections is especially important in the retina because much of the function of this tissue depends on the nature of such connections between cell and cell. In this study also, regional differences can be very significant.

3) Much information of first rate physiological importance can be obtained by the application of various histochemical tests for the detection and localisation of particular substances and enzymes. In these investigations it is often necessary to work with unfixed tissues and by methods which would be regarded with complete abhorrence by the classical cytologists.

4) Parts of the retina and, in particular, the rods and cones and pigment layers have yielded up many of their secrets when subjected to analysis by physicochemical methods, such as the use of fluorescence, of polarised light, and more recently of the electron microscope.

5) Finally, the functional eye is the result of a long evolutionary process, and its behaviour and structure can only be thoroughly understood when seen in relation to this evolutionary history. Both comparative histology in the widest sense, including comparative histochemistry, and a study of embryonic development have therefore important contributions to make in the analysis of retinal function.

These various approaches to the study of retinal structure generally have to be pursued separately since each has its own necessary and appropriate techniques which are often mutually incompatible and possess quite definite limitations. The first question therfore ewhich must be answered by anyone embarking

on a study of retinal structure is "What is the problem which I am trying to solve?" Having decided the answer to this question, then the most appropriate techniques for the purpose can be chosen. Tissues treated in such a way as to show the arrangement of nuclei in the various layers may be quite useless for the purpose of studying the structural basis of the photosensitive mechanism, and procedures which give answers concerning the nature and location of particular chemical constituents may leave the retina in such a state as to be almost unrecognisable from the point of view of micro-anatomy.

It will therefore be the purpose of this section to outline the main requirements in each of these different approaches and to suggest techniques which are appropriate. The techniques can best be grouped under four headings: micro-anatomical methods, cytological methods, histochemical methods and embryological methods, though obviously the lines of demarcation are purely artificial.

—E. N. Willmer.

MICRO-ANATOMICAL AND HISTOLOGICAL METHODS

Special Considerations

1) Impenetrability and general intractability of the cornea, sclerotic coat and lens.
2) Rapid postmortem changes.
3) High lipoid content of the tissue and consequent damage by fat solvents.
4) Ease with which the structures become disorganised and detached from each other.

PRELIMINARY TREATMENT AND METHOD OF FIXATION

For studies of this type, fixed, sectioned and stained tissues provide, in general, the most useful information, though the study of "whole mounts" or flat preparations of the retina is applicable to certain problems, such as the distribution of ganglion cells. In nearly every case, the first problem is to choose a suitable fixative, and the second concerns the method of its application. Even before fixation, however, problems may arise as to how, and under what conditions, to obtain the eye. A few of these may be briefly considered.

1) If the rods and cones are the object of the study, then the effects of the retinal pigment may have to be minimised by keeping the animal (particularly if it be fish, amphibian or bird) in the dark for at least an hour before killing it, in order to allow the pigment particles to be withdrawn into the main body of the cytoplasm of the pigment cells. The animal must be killed and the fixation carried out in deep red light; the eye must then be kept in darkness until the fixative has penetrated, and this may be a matter of an hour or so. Unless this precaution is observed the pigment may very seriously hinder detailed observation, though in reptiles and mammals, where the pigment is less mobile, this does not often occur. It is, however, possible to bleach the pigment (see pp. 178 and 191 ff.) after sections have been cut and before they are stained, though this is obviously not the best treatment for the retina. Albino animals offer definite advantages.

2) Another practical point which may be of importance, even before fixation, is the question of *orientating* the eye when it has been removed from the animal. The very small eyes of very small animals may be fixed in situ in the intact head, and then the whole

head may be sectioned, after decalcification if necessary. In such cases, orientation presents no problem. Similarly, in a large familiar kind of eye, such as the human eye, the orientation can be determined by the stump of the optic nerve and the insertions of the extra-ocular muscles, particularly the oblique muscles. In many other eyes, however, it may be difficult to determine the vertical and horizontal axes, and in such cases suitable marking may be made as follows:

With mouse-tooth forceps a little fold of conjunctiva is grasped just behind the limbus corneae, and with the smallest obtainable curved surgical needle a single stitch of sewing silk is placed in this fold. The meridian chosen for the mark should be at right angles to the intended plane of sectioning so that the microtome knife will never have to pass through the silk.

3) Often there may be no choice as to how the eye is obtained. It may come from an operating theatre or from an animal being killed for some other purpose. When, however, there is choice in the matter, an animal killed by decapitation or coal-gas poisoning is likely to yield eyes in a better state than an animal which has been long anaesthetised, particularly if ether or chloroform has been used, since these may attack the lipoid constituents of the retina, though the evidence for this is not very convincing.

4) With very small eyes and with fixatives which have fair penetrating power the eye may be removed and dropped directly into the fixative. When *removing* an eye from an animal the optic nerve should be divided, close behind the globe, before any tension has been put on it, because pulling on the nerve tends to detach the retina. Care should also be taken not to indent the eyeball. Thus, fine instruments are necessary; human ophthalmic enucleation scissors or manicurist's cuticle scissors, both curved on the flat, should be used. The procedure is somewhat as follows:

With self-locking mouse-toothed forceps a fold of conjunctiva is taken in order to serve as a handle until the eye is finally dropped into the fixative. Behind the forceps the conjunctiva is snipped through and one or two muscles are severed so that the curved scissors can be passed round the back of the eye to cut the optic nerve. With a slight tension maintained on the forceps the eye is then quickly cut away by snipping the remaining tissues of attachment. Any superfluous adherent tissues may with advantage be cut away with the help of the curved scissors, though any final tidy-up may be postponed until the tissue is hardened, and at this stage the eye should be dropped into the fixative as soon as possible. It is often advocated that a small quantity of fixative should be injected with a hypodermic needle into the vitreous body, but the

advantages gained are so small that they do not outweigh the probable damage caused by detachment of the retina. However, the larger the eyeball the more desirable it becomes to make a small puncture in the neighbourhood of the equator. The purpose of this is not so much the penetration of the fixative as the prevention of collapse of the retina and globe consequent on the withdrawal of water from the vitreous body. An unpunctured eyeball will often indent deeply during fixation, but a study of osmotic pressure relationships may help to alleviate this nuisance. The puncture should be made with an ophthalmic discission knife or a knife-needle with a two-edged sword-point, in order to avoid indentation. After the incision has been made, no pressure must be put on the eyeball or the latter will collapse.

5) The methods described produce reasonably good fixation with most fixatives, but intravascular fixation in the anaesthetized animal has many advantages and overcomes the risk of mechanical damage to the unfixed eye. It may, however, introduce other complications in the possible damaging effects of the anaesthetic, and in the upset of the normal tissue fluids in the eye by the preliminary removal of the blood with warm Ringer's solution, before the fixative can be injected. The procedure for mammals follows.

Under Nembutal anaesthesia, open the thoracic cavity by cutting through the ribs on both sides, as laterally as possible, taking care to stop short of the brachial vessels. Cut across the sternum as high as possible. If the heart is beating well, as it should be, there will be a jet from the sternal arteries. This can be stopped with a large pad of cotton wool. Remove the pericardium and put a ligature round the ascending aorta and another round the descending aorta. Make an incision into the left ventricle and insert the cannula through it and up into the ascending aorta, tying it in with the ligature. Tie off the descending aorta. Inject about 50 cc of the appropriate isotonic salt solution, which must contain heparin to prevent clotting; do not exert too much pressure. The injection can be done either with a syringe or from a raised flask. Immediately after this injection has begun, make an incision in the right ventricle to allow a free flow. As soon as all but a small amount of the salt solution has been injected, switch over to the fixative, running in about 400–500 cc for adult cat, rabbit or dog, and correspondingly less for smaller animals. The upper part of the body will then be fixed almost instantaneously. The flasks containing salt solution and fixative are conveniently placed on a shelf 3–4 ft. above the operating table. If the height is less, the fixative does not run through properly; if more, the tap tends to leak and small

vessels may be ruptured. Metal taps tend to leak less than glass ones. The flask of fixative is connected by a rubber tube to a three-way tap also connected to the cannula and to the flask or syringe used for the salt solution. Make sure that the tube is full of fixative and the tap and cannula full of salt solution before starting.

For small mammals, e.g., mice and amphibia (under Urethane anaesthesia) it is more convenient not to tie off the descending aorta but to fix the whole animal. For such animals smaller quantities of both salt solution and fixative are required and syringes can be used for both.

After fixation, dissect out the eyes and place them in the fixative for a suitable period. Cut the eyes in two and remove the lens, etc., before the last change into absolute alcohol.

Four sizes of cannula should be kept, one for adult dogs, cats and rabbits, one for kittens and guinea pigs, one for rats and one for mice. For amphibia a special syringe needle with a knob on the end, to prevent its slipping out, is the most useful. The cannulae must have ridges or they too will slip out.

In cats, where the shape of the orbit and insertions of the extra-ocular muscles make it difficult to get the eyes out without a lot of pulling about, remove the top of the skull and much of the orbital bone before the actual enucleation. In all cases one should work as quickly as possible until the cannula is inserted and the ligatures tied. Don't wait to tie off any vessels that may be cut, just stuff cotton wool over them.

CHOICE OF FIXATIVE

For general histological and micro-anatomical studies, Susa fixative, and mercury-formol (sat sol mercuric chloride, 8 parts; formalin, 40%, 1 part) give good results; so also do fixatives containing potassium bichromate (e.g., Zenker's fluid or Zenker-formol). Neutral formalin (10%; i.e., 4% formaldehyde) may be used for eyes from young animals or for intravital fixation: it should be kept neutral by storage over powdered chalk or magnesium carbonate. The high lipoid content of the retina makes it desirable to use potassium dichromate (3% in water) for 2 or 3 days, even after other fixatives, since this treatment renders the fat less soluble.

For very small isolated eyes, and for more detailed histological and cytological studies of suitably exposed portions of retina, osmic acid or Fleming's solution (2% osmic acid, 5 cc, and 1% chromic acid, 15 cc, preferably without the addition of the usual 1 cc of glacial acetic acid) may be used with advantage, but, on account of their extremely feeble powers of penetration these

fixatives are useless for large eyes, except intravitally, or unless the eyes are previously opened up and steps taken to bring the fixative into intimate contact with the required tissue. This may be achieved by first supporting the eye in a flat-bottomed cup made of paraffin wax, plasticine or some similar material. The eye is then punctured on the equator and carefully cut around in such a way as to detach the anterior half by cautious snipping with curved scissors. Before any attempt is made to lift off the anterior segment the vitreous body must be cut away from behind the lens. The vitreous body can then be removed by carefully sucking it away with a teat pipette, and when sufficient has been removed, the eye, in its container, may be placed in a wide-mouthed glass-stoppered bottle into which 1 ampoule of osmic acid is broken and the stopper applied immediately. After $^1/_2$ hr, enough water is added to the osmic acid to make a 2% solution in which the specimen may stay until next day. This solution, or Fleming's solution, may be used directly in place of osmic vapour, and fixation continued for 24 hr or more. Small pieces of tissue may be satisfactorily fixed in osmic vapour by suspending them over an osmic acid solution by a thread caught between the stopper and the bottle. Care should be exercised in the use of osmic fixatives, since the vapour is very damaging to the conjunctiva and mucous membranes of the investigator.

For the visual cells of fishes and amphibia, Perényi's fluid (24) is recommended, though it is very poor for the retina of birds and mammals and for other parts of the eye. Material should be left in it for not less than 8 hr and for not more than 10, and should be transferred directly to 70% alcohol, which should then be renewed several times within 5 or 6 hr.

Of the bichromate fixatives, Zenker's fluid, "Zenker formol" (Helly's fluid or Maximow's fluid) (7, 24) and Kolmer's fluids are all good, and any one of these may be found best for the tissues of a particular species. Kolmer's fluid has 2 versions, one for cold-blooded and the other for warm-blooded animals. Either fluid is allowed to act for 24 hr, but eyes from large warm-blooded animals profit from an additional 24 hr in the "cold-blooded" mixture. Neither fluid will keep; but stable stock solutions can be made which can, when required, be mixed in equal parts. These solutions are:

For cold-blooded animals:

a)	Potassium dichromate	20 g
	Distilled water	450 ml
b)	Formalin	40 ml
	Glacial acetic acid	100 ml
	Distilled water	310 ml

For warm-blooded animals:

a)	Potassium dichromate	20 ml
	Distilled water	550 ml
b)	Trichloroacetic acid	50 g
	Uranyl acetate	10 g
	Glacial acetic acid	100 ml
	Formalin	40 ml
	Distilled water	360 ml

From Kolmer's fluid the material is transferred to 35% alcohol. A further great advantage of the "cold-blooded" mixture is that it is an excellent decalcifier, and material can be left in it indefinitely. Pathological human specimens may require decalcification; birds, reptiles and fishes often have scleral ossicles, and with small animals the whole head may be treated with this solution. For decalcification the mixture should be frequently made up and renewed. The process then requires only a few days, and the staining qualities of the tissue are unimpaired. Subsequent treatment depends on whether the eye is to be embedded in paraffin or nitrocellulose or is to be cut frozen.

EMBEDDING

For dehydration the tissues are passed through 35%, 50%, 70%, 95% and absolute alcohol. Depending on the size of the eye, the time required in each change is 3–6 hr. Material can, if necessary, be stored indefinitely in 70% or 80% alcohol, but should not be kept in other grades.

If only parts of the retina are to be studied, then only the appropriate region of the eye need be embedded, and embedding offers no special problems; but it may be necessary to preserve the complete relationships of the retina to the rest of the eye, and to include the lens; if so, then special precautions are required. For this purpose any eyeball, excepting one so small that it has been left in the head, needs to be opened widely before embedding if the retina is to be well infiltrated. This is readily accomplished by removing one or two "calottes" at the absolute alcohol stage.

The eye is kept wet throughout the process with a dropper loaded with absolute alcohol, and is supported on a suitable holder, with the intended plane of section parallel to the table. With a new safety razor blade a cut is made with a gentle sawing motion anteriorly and continued towards the back so as to remove a cap-shaped piece (calotte) from the side wall of the globe. The plane of the cut should be deep enough into the globe so that the blade passes very close to the equator of the lens, if not indeed actually through the edge of the lens. A frog's eyeball would need only this one calotte (cap) removed; an eye as large as a dog's

certainly needs to part with a second, diametrically opposite the first, so that a central ring of tissue is all that is left to embed.

If only the retina is required, the lens can be removed, and the eye opened adequately even for nitrocellulose embedding with perfect safety to the retina, by removing the cornea instead of calottes: the whole cornea can be sliced off with a razor blade and the lens hooked out through the aperture with a dissecting needle or very fine (heart) forceps. When a small eyeball has been opened in this way, however, one must thereafter guard against the formation of a bubble within it, which may float it to the surface of any liquid in which it is placed.

After these preparations, the eye goes into clean absolute alcohol if it is destined for nitrocellulose, but goes back into 95% to soften if it is intended for paraffin by way of terpineol or cedar oil.

PARAFFIN EMBEDDING

The use of paraffin for a whole adult eyeball cannot be advised, owing to the hardness of the fibrous tunic, to say nothing of the lens. Good sectioning in paraffin is possible only with pieces of isolated retina. The globe wall, in absolute alcohol, may be cut up with a razor blade and each piece of retina isolated with fine instruments.* The original location, and the orientation, of the piece can be identified from its shape, made distinctive if necessary by chipping off a corner. It is better to fold the end of a strip of stiff paper and use this, rather than forceps, to transfer the piece from fluid to fluid.

Embedding is best carried out in a heated watchglass containing a round coverslip, both thinly smeared with glycerin. The piece of tissue is placed, convex side up, on the coverglass with a warm needle. When the coverglass is later split away from the block it leaves a surface so polished that the tissue can be seen clearly and orientated precisely on the microtome.

The investigator whose problem seems to demand serial sectioning may decide to use paraffin, instead of nitrocellulose, for whole-eye sections. For his benefit, there are certain departures from the ordinary paraffin method which may be suggested. Singly or in combination they may make the difference between discouragement and elation. They are: *a*) the use of terpineol, *b*) the use of rubber-paraffin, and *c*) softening before cutting.

a) *Terpineol* (lilacene, "synthetic oil of lilac") is valuable for clearing. Material goes from 95% alcohol (or even 80%) directly

* Since the choroid is soft, one should never risk damage to the brittle visual cells by attempting to separate the choroid from the retina if the choroid more readily separates from the sclera.

into terpineol (and *can* remain there indefinitely). After 24 hr, it is transferred to clean terpineol. Some shavings of paraffin are added, and the vial is left at a temperature well below the melting point of the paraffin until the terpineol seems to have taken up all that it will.

The specimen is now transferred to melted paraffin in a small clean vial, being allowed to drain quickly but carefully on paper toweling on the way. The procedure from here on may seem weird, but it is dictated by the fact that terpineol is nonvolatile. The specimen is transferred to a clean vial of fresh paraffin every hour or so until no odour of terpineol can be detected. Then, and only then, can it be embedded. It is fatal to try to use one vial, pouring off the old paraffin and pouring in the new, as the inevitable creepage on the outside of the vial will retain the lilac odour indefinitely. The time required is determined partly by the size of the specimen, but largely by the frequency of the changes of paraffin. The removal of all terpineol may require several days for a large eyeball, but the specimen will take no harm from so long a stay in the embedding oven if it has been through terpineol.

b) Rubber-paraffin will hold the fibrous tunic together much better than ordinary paraffin, however hard, and may even permit good sectioning of the lens in some kinds of eye (provided that the lens capsule has been nicked in the absolute alcohol, to let the colloidal rubber get in). A stock solution of 53–55° C paraffin, saturated with crude rubber, is prepared. When several specimens are to be embedded the procedure is as follows: Mix 720 g of paraffin wax (M.P. 53–55° C) with 80 g of either bayberry wax or white beeswax and 5 ml of aniline, and stir in from 140 g (in winter) to 160 g (in summer) of the rubber stock.

c) Softening.—To soften the hard tissues in paraffin blocks so that they will section more smoothly, the block may be shaved away, deeply enough to cut *into* the tissue, in a direction parallel to the intended plane of section, and the chucked block placed in a mixture of 9 parts of 60% alcohol and 1 part of glycerin. Overnight may be long enough, but only experience will determine the time required by a given piece.

NITROCELLULOSE EMBEDDING

This method has great advantages for the preparation of good material from the eye. The nitrocellulose penetrates the tissues well, supports them well and allows large and thin sections to be cut. It should, however, be remembered that all the reagents are extremely inflammable. Numerous modifications of the process have been described (7, 21, 25, 116).

The 20% stock nitrocellulose* solution is made up as follows (21):

Nitrocellulose	140 g
Absolute alcohol	210 ml
Ether	250 ml
Celloidin (plasticiser)	4 g

The celloidin is first dissolved in alcohol and ether and the nitrocellulose added when it is completely dissolved. It is allowed to stand for some time and then decanted into and stored in a wide-mouth glass-stoppered bottle with a well-fitting clean ground-in stopper.

The specimen to be embedded is left for at least 24 hr in 5% nitrocellulose made by diluting the 20% solution with a mixture of equal parts of alcohol and ether. It is transferred to 10% nitrocellulose for 24 hr or more and then to the 20% solution for at least 4 days. Time spent in the weaker concentrations is important in determining the quality of the infiltration, and these times are given on the assumption that the eye has been opened up in a manner similar to that described in the preceding section, and that excess fluid is drained away from the cavity before transference to the thicker solutions. The specimen is placed in a paper box, allowing a margin of about 10 mm all around the specimen and about 20 mm above. Nitrocellulose, 20%, is poured over it. The box is then placed in a large desiccator and allowed to remain undisturbed for about 12 hr, by which time all air bubbles should have disappeared. By raising the lid of the desiccator slightly by a thickness of paper, evaporation from the nitrocellulose block can be allowed to proceed slowly, and if the surface of the block gets too hard it may be softened slightly with a mixture of equal parts of ether and alcohol and then more 20% nitrocellulose added. The block is then left until it has only a slight "give" when the box is pressed between the fingers. This may require several days and depends on the conditions of temperature, etc. The box and block are immersed in 75% alcohol, and the alcohol is renewed 3 times in a period of 2 days. Alternatively, the block may be hardened in chloroform. The paper can then be removed and the block trimmed, and fixed to a completely dry wooden microtome specimen holder with 20% nitrocellulose. This nitrocellulose is hardened in the air, and the whole block then immersed in 75% alcohol. During the hardening, the actual block should be protected by a pad of cotton wool immersed in 75% alcohol. Again, chloroform may be used for solidify-

* The nitrocellulose may be obtained from various dealers; e.g., from Edward Gurr, 108 Waterford Road, London, S. W. 6, and is described as Industrial nitrocellulose damped with 7:3 B.A. HX 30/50; or from Hercules Powder Co., Gillespie, N. J., as RS 1/2 sec low viscosity nitrocellulose 30% solvent in absolute alcohol.

ing the nitrocellulose and the block may be kept in chloroform. The block is then cut on either a sliding or rotary microtome, preferably the former, being kept moist with 75% alcohol periodically applied. The sections are placed in 80% alcohol and can then be stained as desired.

The stained sections can be flattened on the slide in 96% alcohol, by covering with a piece of thick toilet paper or cigarette paper and gently pressing out with a glass rod. They are washed with a mixture of equal parts of alcohol and chloroform or with isopropyl alcohol and again pressed out with a glass rod over toilet paper, the process being repeated several times. Finally, several applications of xylol are made in the same manner and the section is mounted in balsam or a neutral mounting medium. Slight pressure should be maintained on the coverslip until the balsam is hard. A thin disc of cork may be clamped over the coverslip, with a spring clothes peg, and the slides stored thus in the paraffin oven. If a little albumen is used to attach the sections to the slide, as for paraffin sections, the nitrocellulose may be removed by dissolving it in oil of cloves, but if the nitrocellulose is solid enough it is best left alone, and it does not stain appreciably.

Section cutting may be made easier by immersing the chloroform-hardened block in terpineol for two or three days; though, if the terpineol is allowed to act for very long, the block may then become too soft and the sections will pucker, so that the tissue may have to be dissolved out and re-embedded. The block, thus softened, may be cut "dry." If the lens is still present, the block should be orientated in such a way that the required part of the retina is not cut by the same part of the knife as cuts the hard lens. Great care should be taken in orientating the block so that the sections contain the desired structures orientated in longitudinal or transverse directions.

If infiltration has not been complete, some of the less permeable structures may shatter away from the sections. This can be remedied to some extent by painting the block, after cutting each section, first with 2% nitrocellulose and then with chloroform, and immediately cutting the next section with a knife flooded with chloroform. Very thin sections may be obtained by this method.

STAINING, ETC.

Nitrocellulose sections, whether cut dry or wet, will have come off the knife into a dish of 80% alcohol in which they flatten out. This fluid, if contaminated with terpineol, should be carefully decanted or pipetted off and replaced by fresh 80% alcohol. Material which has been fixed with mercury-containing fixatives is treated

at this point by dropping a flake of iodine into the dish; it may be transferred to 95% alcohol when the iodine has removed the mercuric deposits. Sections can stay in 95% alcohol almost indefinitely until time is found to stain them. If they do become a little soft, they can be hardened again by a short sojourn in 80% alcohol. If bleaching of the pigment is necessary, this is best done by Mayer's "nascent chlorine" method (see Bolles Lee (59)), or in a dish of 70 or 80% alcohol in which 1 drop of bromine has been dissolved. Diaphanol, though more convenient, puckers nitrocellulose sections and badly interferes with staining.

In handling unattached sections, one should avoid staining procedures which call for brief periods timed to the second, because one cannot stain whole dishes full of sections at once. If one is content to stain one section at a time, this is best done by transferring the section from bath to bath on a stainless metal section lifter, holding the section on by one corner with a dissecting needle. Alternatively, one may prepare strips of stiff letter paper for lifters, and pin a corner of a section to such a strip by pushing a slender insect pin vertically through both (on a wood surface). The section can then be snatched out of an opaque stain on the second, with no hunting for it.

Staining sections en masse involves decanting each fluid and pouring in the next, with the sections remaining in the same dish. One may also devise baskets of metal cloth which form close-fitting inserts for large dishes, and carry the sections from dish to dish. No intermediate alcohols are needed in passing between 95% alcohol and water in either direction, but the water should contain a trace of detergent or 1 drop of liquid soap so that sections entering it from alcohol will not scoot about on the surface and tear or fold themselves.

Sections obtained by these methods can be relied upon to show much of the anatomical structure with very little distortion, and the sections can be stained by a variety of methods, whose effectiveness depends largely on the fixative used in the first place.

For general purposes, *Heidenhain's iron haematoxylin stain*, which is best employed regressively (mordanting and staining for 12–24 hr each), is an excellent stain for the retina.

Mordant	Ferric ammonium sulphate (violet crystals only)	40 g
	Sulphuric acid	1.2 ml
	Distilled water to	1000 ml
Stain	Haematoxylin	5 g
	Distilled water	1000 ml

The haematoxylin solution should be kept to "ripen," or it can be used directly if 1 g of potassium iodate (KIO_3) is added. If

the mordant is diluted with an equal quantity of distilled water it can be used as a satisfactory differentiator. Each nitrocellulose section or slide of paraffin sections is best treated separately by this technique, and the times altered to give the required amount of staining. Counterstaining of nitrocellulose sections is best done with alcoholic solutions of eosin, followed by a quick wash in 95% alcohol before transferring to absolute alcohol and chloroform.

Mallory's triple stain, Heidenhain's "azan," Mallory's phosphotungstic acid haematoxylin, Masson's trichrome (7) and Held's molybdic haematoxylin (59) all produce well-differentiated pictures of retinal structure.

Mallory's triple stain can be used as a rapid test stain or in more permanent form. For rapid staining, the following solutions are required:

1.	Acid fuchsin	0.1 g
	Distilled water	100 ml
2.	Phosphomolybdic acid	1 g
	Distilled water	100 ml
3.	Orange G	2 g
	Aniline blue	0.5 g
	Oxalic acid	2.0 g
	Distilled water	100 ml

Heat this solution until it steams; cool and filter.

A nitrocellulose section requires the following times. Solution 1—20 sec, rinse in water; solution 2—2 min, rinse in water; solution 3—45 sec, rinse in water; 95% alcohol until the nitrocellulose is free of blue. Absolute alcohol and chloroform. Clear in xylol and mount in balsam.

For more permanent staining the solutions are:

1.	Acid fuchsin	2 g
	Distilled water	750 ml
2.	Phosphotungstic acid	7.5 g
	Distilled water	750 ml
3.	Orange G	15 g
	Aniline blue	3.75 g
	Phosphotungstic acid	7.5 g
	Distilled water	750 ml

The staining times are now much more flexible and depend on the fixative and material. The limits are: *1*) 1–5 hr, rinse in water; *2*) 6–12 hr, rinse in water; *3*) 3–6 hr, rinse in water, followed by 95% alcohol until the nitrocellulose is colourless.

Azan staining is another very satisfactory and less time-consuming modification. The solutions required are:

1) Azocarmine: Bring 0.1% azocarmine in water to boiling point and allow to cool, filter through wet filter paper and add 1 ml of glacial acetic acid

2) Aniline alcohol: Add 1 ml of aniline to 100 ml of 98% ethanol
3) Acetic alcohol: Add 1 ml of glacial acetic acid to 100 ml of 98% ethanol
4) Phosphotungstic acid: 5 g in 100 ml distilled water
5) Aniline blue-orange G: Dissolve aniline blue 0.5 g and orange G 2 g in 100 ml of distilled water; add 8 ml of glacial acetic acid; bring to boiling point, cool and filter cold; dilute 1–3 times with distilled water

Stain in *1*) at 60° C for 10–15 min. Rinse in distilled water, differentiate in *2*) until the nuclei stand out clearly. Treat with *3*) for 1 min; with *4*) for 15 min, rinse in distilled water and stain in *5*) for 5–15 min. Wash in distilled water, differentiate in 90% ethanol, dehydrate, clear and mount.

A lengthy Heidenhain's haematoxylin stain can be combined (without differentiation) with solution *3*) of the permanent Mallory's stain, or with solution *5*) of the azan stain. Enough of the haematoxylin will remain in the chromatin to make the nuclei particularly crisp in appearance, instead of glassy, as when only acid fuchsin or orange G has stained them. Mallory's stain is satisfactory after any "dichromate" fixative, but is unsatisfactory after osmic acid. Heidenhain's iron haematoxylin is particularly satisfactory after the latter.

Kolmer finds that Unna's water-blue-orcein-safranin stain for epithelial fibres gives differential staining of two types of cone in primate retinae (54). This differentiation is also observed with Mallory's stain, azan, Heidenhain's iron haematoxylin and Held's haematoxylin (58). It is probable that the fixative is an important factor in obtaining this differentiation. Susa and mercury-formol are certainly effective.

CYTOLOGICAL METHODS

Special considerations

1) Micro-anatomical relationships are relatively unimportant.
2) True structure must be differentiated from artifact.
3) Localisation of precipitates and colour reactions must be considered in relation to the rates of diffusion of the reacting substances and to the availability of suitable surfaces for precipitation, etc.
4) Allowances must be made for possible postmortem changes.
5) In making measurements, or in determining the spatial relationships between cell structures, allowances must be made for such artifacts as may be caused by shrinkage, swelling and by metallic precipitation. Nerve cell processes are probably amœboid in life; they certainly are in tissue culture. Fixation often causes these processes to become globular.

METHODS FOR GENERAL CELL STRUCTURE

These distinctions in the approach to the study of the retina are clearly somewhat arbitrary and the methods must overlap to a greater or less extent. It is probably helpful, however, to emphasise the different disciplines, and under this heading of cytological methods the essential structure of rods and cones, and of the other cell types in the retina will be considered, and less attention need now be paid to the preservation of the intact structure of the eye or retina as a whole. Provided that the cells in question are well seen and under controlled conditions, other considerations are irrelevant. Some of the methods applicable to micro-anatomy are of course equally applicable to the cytological study, and they can also be applied to smaller pieces of tissue isolated by careful dissection. Osmic fixation is ideal for many studies of cytological structure and is particularly suitable for those with the electron microscope. Post-chroming with 3.5% potassium dichromate is also very beneficial. Iron haematoxylin staining is again excellent. It is clear, however, that both paraffin and nitrocellulose embedding must destroy many of the properties of the cells and tissues, because lipoid constituents are initially abundant in the living retina and both methods involve the use of strong fat solvents.

Many of the cytological techniques are therefore better applied to frozen sections or to living tissues which have been teased apart in aqueous humour, or serum. Teasing in Ringer's solution alone is not very satisfactory. Material freshly teased in serum can be

studied immediately by the direct vision, dark field, phase contrast, polarising, reflecting or ultraviolet microscope, or alternatively it may be fixed and stained as required, or spread on cellophane sheets and dehydrated for examination with the electron microscope (99). It must be remembered that such living teased material rapidly undergoes changes, even in the best media, and prolonged study in the unfixed condition should be avoided when the normal structure in the animal is under consideration. Furthermore, in interpreting pictures obtained by the electron microscope, the effects of complete dehydration on the complex architecture of a protoplasmic system must be weighed in the balance. On a more gross scale, plums and prunes have certain very evident differences but in comparison with tissues under the electron microscope the latter are hardly desiccated at all. Nevertheless, work (26c, 26d, 100, 101) on osmic acid-fixed rods and cones, which have then been embedded in *n*-butylmethacrylate and cut into extremely thin sections and examined by the electron microscope has yielded some very important data not only on the essential structure of the visual cells but also on certain differences between the architecture of the outer segments of rods and cones and on the probable mitochondrial nature of the ellipsoids. For the details of the methods used, the original papers should be consulted.

These results certainly confirm the observations of Lewis (60) obtained by vital staining (Janus green B) and other observations made by earlier developed techniques. To demonstrate the mitochondrial nature of the ellipsoids, and perhaps to some extent also of the outer segments of rods and cones, the following method (16) gives successful permanent preparations.

PERMANENT PREPARATIONS

1) Fix the retina in Helly's fluid for 6 hr.

2) Post-chrome for 48 hr at 37° C in a saturated aqueous solution of potassium dichromate.

3) Wash overnight in running water.

4) Embed in paraffin wax and cut sections of a suitable thickness (3μ). Bring the sections down to water, treating with iodine in 70% alcohol and then in 5% sodium thiosulphate on the way.

5) Dry the slide except where the sections are, flood with 20% acid fuchsin in aniline water (i.e., water saturated with aniline) and heat gently until steaming, in order to overstain.

6) Wash off the acid fuchsin with distilled water, observe the section under the high power of the microscope and irrigate with an alkaline solution. One drop of the saturated aqueous solution

of sodium bicarbonate in 10 ml of distilled water gives a fairly rapid differentiation.

7) To stop the differentiation and brighten the acid fuchsin, dip the slide in 1% HCl. If brightening is undesirable because extraction of the dye from the cytoplasm is difficult, wash in distilled water instead of dipping into the acid.

8) When the slide has been correctly differentiated, wash it in distilled water, counterstain progressively in ½% aqueous solution of methyl blue. Wash off the stain with distilled water, dip in 1% HCl for 3 sec only, wash again in distilled water, go through the alcohols without any hurry, clear and mount in balsam.

By a combination of histological and cytological methods, information can be obtained as to the dimensions, distribution and general cyto-architecture of the cells. The properties of the inner and outer segments, the ellipsoids, paraboloids and myoids of the receptor cells can be investigated. Good fixation (osmic acid or Susa fluid) reveals differences between rods and cones and distinguishes, mostly on the appearance of the nuclei, between horizontal cells, amacrine cells, bipolar cells, the cells of Muller's fibres, and between at least 2 groups of ganglion cells; but the study of fixed material inevitably leaves many questions unanswered with regard to the actual construction of the cells.

METHODS FOR SYNAPTIC CONNECTIONS

One of the main problems in retinal physiology lies in the elucidation of the spatial relationships between various types of cells. For example, it is essential to know the exact synaptic connections between the rods and cones on the one hand and the bipolar cells on the other, and again between the bipolar cells and the ganglion cells. Methods which have so far yielded results in this direction fall into four categories: *1)* the Golgi methods, *2)* other silver impregnation methods, *3)* methylene blue methods and *4)* the use of the electron microscope on osmic fixed material.

Some of the most important factors in bringing about successful metallic impregnations are the chemical cleanliness of all glassware, the use of glass-distilled water, prevention of contamination by dust, and the use of glass for manipulation of the sections and the avoidance of contact between the tissues and metal of any sort.

GOLGI METHODS

These metallic impregnations were extensively used by Cajal [17, 18] and more recently by Polyak (84), and the reader is re-

ferred to *The Retina* by Polyak for full information. The methods have the extraordinary quality that they impregnate only a few cells in any one preparation, and there seems as yet to be no explanation for this selective action. Moreover, those cells which are impregnated tend to be covered with the precipitate completely and along all their processes, but the precipitate does not extend to the next cell. Unfortunately one can never be quite certain of this completeness, and this may sometimes provide a serious source of error in interpretation. For example, does the apparent absence of an axon really mean that the cell does not possess one? Certainly the majority of the cells stained at all seem to be stained completely, but some doubt inevitably remains. On the whole, however, the methods are very satisfactory for studying the nature of the processes belonging to the different cell types, though they can but rarely give an answer to the question of *which* other cells, and never to the question of *how many* other cells are involved in the synaptic connections. The methods are very capricious and depend on many unknown factors. Failure with one attempt does not always mean that the procedure will inevitably fail.

There are two rather distinct procedures: the Golgi technique and the Golgi-Cox technique. Both are more reliable when the tissue is taken from young animals and, in both, it is essential that the tissues should be placed in the fixing solutions as soon as possible.

Golgi procedure

1) Fix the tissue for several weeks in:

Potassium dichromate	2 g
Sodium sulphate	1 g
Distilled water	100 ml

Impregnate in 1% silver nitrate for several days, if possible removing a part of the material on each successive day, for subsequent sectioning. Sections should be cut thick (>20μ), and this may be conveniently done on a freezing microtome. They should be mounted in pure balsam (7) without a coverslip. When the balsam is dry, a coverslip can subsequently be placed on the preparation and by the application of heat and gentle pressure pressed into contact with the balsam.

2) Fix the tissue in:

5% Potassium dichromate	1 part
2% Osmic acid	1 part

Both these solutions should be made up in double-distilled water and all glassware should be chemically clean and free from dust. The solutions should be mixed just before use. Only small pieces of

tissue less than 3 mm thick should be used and fixation should continue for 2–5 days, depending on the size of the pieces. The fixative may be introduced with a hypodermic syringe into the eye provided that an exit hole has been previously pierced through the opposite side of the eyeball with a sword-point needle. If 2 or 3 injections are made during the first 15 min after the eye has been removed and placed in the fixative, the eye can be opened up and as much as possible of the vitreous humour removed before it is left to soak in fresh fixative for 2–5 days. (For the reasons given earlier, the osmic vapour should not be allowed to escape too widely.)

After fixation, the tissue is drained and then placed in 0.75% silver nitrate solution in distilled water. The silver is renewed until it no longer goes cloudy and yellow, and the tissue is left in it for 2 or 3 days. Before embedding, any excessive precipitation on the outside of the tissue may be carefully scraped away with cotton wool pads.

Celloidin or nitrocellulose embedding is preferable to paraffin embedding, as the latter causes shrinkage which obviously interferes with any interpretation of the synaptic connexions.

Golgi-Cox Procedure

In this procedure mercury takes the place of the silver. Fix and impregnate the tissue in a solution made up as follows:

Mix 10 g of potassium dichromate dissolved in 450 ml of distilled water with 10 g of mercuric chloride in 450 ml of distilled water. A solution of 3 g of potassium chromate in 100 ml distilled water is then slowly added. The fixative may be conveniently applied by injection as outlined above, followed by opening up the eye. The method is also applicable after formalin fixation, and the impregnation should be allowed to continue for more than 2 months. Thick sections are then cut by the celloidin or nitrocellulose technique and the sections are treated with 5% sodium sulphite in distilled water for about an hour before being dehydrated and mounted in the manner described for other Golgi sections.

OTHER SILVER IMPREGNATION METHODS

Numerous other silver impregnation methods have been applied to the central nervous system and many to the retina. These mostly bring out the "neurofibrils" and finer processes of the nerve cells. Since they are not so selective as the Golgi methods, far more cells are impregnated and thick sections cannot readily be used in which to trace the ramifications of the cell processes because these get lost in the dense mass of other fibres. However, in

one or two of the methods, some types of cells are impregnated more heavily than others, and much depends on the conditions of fixation for the particular features which are presented by any given piece of tissue. The hydrogen ion concentration, at the time of fixation and subsequently, is one factor of very considerable importance. Traces of other heavy metals, as in the Bodian "activated" protargol technique, where metallic copper is added to the silver albumose solution, increase the selectivity (9). In the case of the eye, the method of Nonidez (78), which is a modification from Cajal's original methods, produces good impregnation of the horizontal cells and of the large ganglion cells. The smaller ganglion cells remain practically untouched.

Nonidez Procedure

Fix for 24 hr to 3 days in

Chloral hydrate (crystals)	25 g
50% ethanol	100 ml

Remove excess fixative with filter paper and transfer to

95% ethanol	100 ml
Ammonia S. G. 0.896	6 drops

Renew this mixture if much tissue is being treated. Leave for 24 hr.

Rinse with distilled water (5 min) and transfer to 2% silver nitrate solution in distilled water for 5–6 days in the dark at 37–40° C. Renew the silver after 2 days or when yellowish brown.

Rinse with distilled water 2–3 min, and reduce the silver for 24 hr in

Pyrogallol	2.5–3 g
Formalin	8 ml
Distilled water	100 ml

Wash in several changes of distilled water for 2–3 hr and transfer to 50% ethanol and change this twice in the next 24 hr.

Dehydrate and embed in celloidin, nitrocellulose or paraffin. Amyl acetate is recommended as clearing agent and paraffin solvent.

It is of interest to observe that when this method is applied to ganglia, the postganglionic fibres of parasympathetic ganglia impregnate much more heavily than those of sympathetic ganglia, so it is probable that some difference in microchemical structure is being revealed by this method. Horizontal cells are also brought out by the Bodian technique (10), by the methods of Holmes (48) and indeed by many silver techniques. In the retinae of birds certain amacrine cells are also clearly impregnated by these methods (35, 111).

Palmgren Silver Method

The large ganglion cells and nerve fibres are also well shown by the silver method of Palmgren (82), when this is applied to retinae fixed in Bouin's fluid and stretched out flat on a slide with the ganglion cell layer uppermost. This method has proved particularly valuable in relating the probable source of the retinal action potentials, as so far investigated, to the large ganglion cells (88). The smaller ganglion cells are also impregnated, but less heavily. The method was originally devised for the rapid and selective staining of sections of nervous tissue on the slide.

Solutions required:

1)	1% Nitric acid		
2)	Glycine	5	g
	Distilled water	20	ml
3)	Formalin (comm)	25	ml
	Distilled water	75	ml
	1% Nitric acid	0.2	ml
4)	Silver nitrate	15	g
	Potassium nitrate	10	g
	Distilled water	100	ml
	Glycine solution (2)	1	ml
5)	Pyrogallol	10	g
	Distilled water	450	ml
	Abs ethanol	550	ml
	1% Nitric acid	2	ml
6)	Gold chloride	0.5	g
	Distilled water	100	ml
	Glacial acetic acid	3	drops
7)	50% ethanol	100	ml
	Aniline	2	drops
8)	Sodium thiosulphate	5	g
	Distilled water	100	ml

Wash the retina in solution *3*) until no more yellow colour comes away, and spread it out on a slide. Cover with the silver solution *4*) for 15 min at 20–25° C or 4–5 min at 35°. Run off the excess of silver and replace directly by solution *5*) for 1 min at 35–45°. Wash it with this solution several times. Rinse in 50% ethanol for 10 sec. Wash in distilled water and wipe off any silver precipitate from the slide. Examine under the microscope and repeat the whole process if necessary. Tone in solution *6*) until the yellow colour disappears and, without rinsing, place it for 15 sec in solution *7*). Wash in water and fix the silver for 5 sec in solution *8*). Rinse, dehydrate and mount in balsam.

VITAL STAINING METHODS

The localisation of ganglion cells can also be achieved in certain fish, and perhaps in other animals too, by the application of

1:10,000 neutral red solution in the appropriate Ringer's solution to the opened eye.

The ganglion cells and their processes can also be investigated by vital staining methods involving methylene blue and related dyes. These again are perhaps most usefully applied to the retina as a whole, when it is spread out flat with the ganglion cell surface uppermost. Methylene blue can also be used by injection into the anaesthetised animal (84).

Methylene Blue Injection

Staining solution:

Vital methylene blue	0.05 g
Resorcin	0.01 g
Dextrose	0.2 g
NaCl	0.72 g
Water to	100 ml

Procedure.—Make a hole in one angle of the eye in the anaesthetised animal a few millimeters behind the corneal limbus, avoiding any considerable extra- or intra-ocular haemorrhage. With the point of a needle make a few carefully executed movements in the substance of the vitreous humour in order to rupture its membranes and threads, taking care not to damage the posterior surface of the lens or the retina. Inject the methylene blue solution directly into the vitreal cavity by piercing with a fine sharp hypodermic needle in the opposite angle of the eye, using a very slight pressure only. About 0.5 ml of the solution is injected in about 5 min, the determining factor being the pressure encountered. The animal is allowed to live for about 30–40 min after the injection.

Remove the eye from the orbit, open it by a circular cut behind the sclero-corneal junction and place the posterior part of the eyeball in a small beaker containing enough saline solution to permit complete submersion of the specimen. Divide the specimen into smaller segments and separate the retina gently from the other coats. Change the saline solution in order to keep the specimen from being soiled by blood and pigment and to prevent postmortem staining of the retina. Cut off parts of the vitreous body.

Mount the retina on a slide, avoiding rolling up of the specimen and with the vitreal side up. Suck off all the superfluous fluid and keep the slide in a Petri dish with lid on to prevent too rapid drying. A few drops of saline solution may be added from time to time, if necessary. The specimen remains exposed to air, in the covered Petri dish, which is placed on a hot plate at 40° C. Allow the blueing to develop fully and observe periodically under the microscope.

Fix the tissue in a solution of 95 ml of a 10% ammonium molybdate and 5 ml of glacial acetic acid for 1–2 hr in the refrigerator. Wash quickly but thoroughly in several changes of ice-cold distilled water to remove the fixative, and dehydrate the specimen with ice-cold absolute alcohol in the refrigerator. Clear with cedarwood oil (not clove oil or carbol xylol) and cover with Canada balsam; dry rapidly on a hot plate at 40° C. Cover with a coverslip by slightly increasing the temperature and pressing the surplus balsam out from underneath the coverslip. Carry on the drying of the slide on the hot plate for 3 days.

HISTOCHEMICAL METHODS

Special considerations

1) The specificity of the reactions must be determined.
2) The design of suitable control tests is of paramount importance.
3) Localisation of precipitates or colour reactions must be considered in relation to the rates of diffusion of the reacting substances and to the availability of suitable surfaces for precipitation.
4) The permeability of the cells and tissues to the reagents may be a limiting factor.
5) The effect of altered conditions, of fixatives, etc., on the activity and localisation of enzymes, etc., is important.
6) Failure to detect a substance does not necessarily mean the complete absence of that substance from the living tissue.

The methods which have so far been described are mostly concerned with the study of the retinal cells in their proper relationships with each other, their relative numbers, position, arrangement and architecture. This, however, is only one side of retinal cytology and, in what follows, it will be seen that considerable sacrifices can be made in respect of the standards of classical cytology in order to elucidate the intimate processes which go on in the retina. Essentially these processes must involve a photochemical change or perhaps several different photochemical changes, which are made to initiate impulses along particular neural pathways, and so to provide the brain with information from which it may build up a picture of the outside world in colour, shape, light, shade, movement and all the other attributes of vision. It is the task of the retinal histologist or cytologist to examine how these mechanisms function, and the study of the protein skeleton of the retina, which results from "fixation" in the classical sense, will not assist beyond a certain point. The photochemical processes must be investigated on a cellular level and ultimately on a submicroscopic and molecular level; so also must the nature of the pathways and the mechanisms of transmission from cell to cell. Various useful cytochemical tests can be successfully applied to retinal cells and even to the retina as a whole, but the price to be paid is often heavy in terms of structural organisation. It may be necessary to tease out the retina in serum, or even to cut frozen sections of the fresh unfixed tissue entirely untreated, or to cut it in 25% gelatin (80) in water (if acids are not to be used subsequently) in order to investigate the nature of pigments or enzymes, and it

may be impossible to make "permanent" preparations. These, however, are mere details if, in spite of them, the substances in question can be correctly located and identified.

VISUAL PIGMENTS

If a frog's retina is teased out under red light in serum, Ringer's solution or aqueous humour, after the animal has been kept in the dark for several hours, and it is then examined under the microscope and the field illuminated with white light, the outer segments of the majority of the rods can, for a short time, be seen to be distinctly reddish (11). The colour quickly fades, only to reappear rather less intensely after a period in the dark. This clearly shows that the visual purple is present in the outer segments and can be bleached and regenerated in the absence of any other elements in the vicinity; i.e., the pigment epithelium is not, as at one time supposed, a necessity for the immediate regeneration of the visual purple, though it may be involved in other ways.

The density and the spectral sensitivity of the photosensitive pigments in the visual cells in the frog have been investigated both for the retina as a whole and also for individual rods. For the latter purpose, the following method (26) is satisfactory. It is essentially a photographic method and is based on a comparison of the densities of the photographs of individual rods under different conditions of illumination.

Photographic Method for Individual Rods

The retina is dissected in dim light (usually red) under Ringer's solution. It is floated into a well upon a microscope slide, the well being made by fixing a ring of wax upon the slide. The well, filled with Ringer's solution, is covered by a coverslip to prevent the preparation from drying.

The microscope light source is a tungsten filament lamp underrun at an accurately controlled voltage. The microscope is usually used with a $^{2}/_{3}$ inch objective and a 10× eyepiece. The photographs are taken upon 35 mm H.P.3 film. It is important not to try to use too high a magnification as so much light then has to pass through the preparation to take a photograph that the photosensitive pigments in the retina are appreciably bleached.

A holder for colour filters is mounted in front of the microscope light source. A holder for neutral filters is placed between the mirror and the substage condenser of the microscope. The neutral filters are therefore in the almost parallel beam of light coming to the microscope substage diaphragm. (Their density would be different if placed in a converging or diverging beam.) The flashes

of light used last about 10 sec and are given by a slowly rotating shutter between the light source and the microscope.

The retina is focused in dim light (usually red). Great care is taken not to expose it, before bleaching, to light other than the minimum required for focusing and making photographs.

The method for measuring the density change of individual rods after bleaching at one given wavelength is best described in terms of one experiment.

Three photographs are made before bleaching. The photographs differ only in that neutral filters of density 0.1, 0.2 and 0.3 are placed in turn beneath the substage condenser. The preparation is now bleached for 2 min in the strong white light obtained by taking away both colour and neutral filters. This light is so bright that it bleaches the preparation completely in a few seconds. Ten minutes after bleaching, photographs are made with neutral filters in the series of densities 0.1, 0.2, 0.3, ... 1.5.

The film is developed and enlargements are printed. Prints to be compared must be made in exactly the same way and must come from negatives on the same roll of film which have been developed at the same time.

From the prints it is possible to see what change in neutral filter exactly replaces the density change caused by bleaching. If this density change upon bleaching were, for example, 0.5, then the rods on the photograph made with the neutral filter 0.1 before bleaching will match those made with 0.6 after bleaching. Similarly, 0.2 before bleaching will match 0.7 after bleaching and 0.3 before bleaching will match 0.8 after bleaching.

The comparisons are made by cutting across a line of rods in a print of retina before bleaching, and laying the half rods at the edge of the cut against their corresponding halves on the prints of retina after bleaching. It is usually easy to compare the same individual rods before and after bleaching. The two halves of rod to be compared are isolated with a small diaphragm from their backgrounds and examined under a low power microscope.

Photographic Method for Whole Retina

By means of a photoelective densitometer and the same photographs it is possible to compare the average density changes on bleaching the retina as a whole with that obtained by the foregoing method for the individual receptors. The average change can thus be obtained more accurately but the effects occurring in individual cells are, of course, lost. The method can also be used to determine the approximate spectral sensitivity curve of the principal pigment present.

A calibrating series of photographs of the bleached retina is made at one wavelength (usually $\lambda = 0.52\ \mu$). Photographs are then taken before and after bleaching for a series of colour filters covering the spectrum to be used. In this way the density changes caused by the lights transmitted by a series of filters can be found and a curve showing density change against wavelength can be plotted. The change in contrast of film for lights of different wavelengths may have to be considered in the interpretation of results.

In these methods the rods and cones are examined in situ and the light passes along their long axes. In the following method which has been used (111) for measuring the actual absorption of light of different wave lengths by individual cells in the toad (*Xenopus*), this advantage is lost, but the absorption spectrum can be obtained before and after bleaching. Observations with polarised light (see p. 196) indicate that the orientation of the visual cells with respect to the direction of the light may have some significance.

Measurement of Absorption of Light

Decerebrate the animal in deep red light; puncture the cornea with sharp scissors; cut off the cornea, remove the lens and lift the retina out gently with very fine forceps. Place the retina in 1 or 2 drops of serum from the same species and cover with a coverslip. There should be enough serum to prevent drying under the edges of the coverslip but not enough to allow the specimen to float about. A number of rods (but by no means all) and a few cones will be detached and lying on their sides. Choose a suitable rod under the microscope with red light and get it into accurate focus. It is an advantage to have the focus roughly right beforehand. Photographs are then taken of the rod with lights of different wavelengths. About five exposures are made on 1 plate, beginning and ending on each place with a reference wavelength usually near the point of maximum absorption to check on possible bleaching. The exposure for each wavelength is adjusted to give the same density of background every time, remembering that the serum itself is slightly yellow so that the short wavelength exposures will be a trifle longer than with a colourless background.

A calibrated neutral wedge is photographed alongside the rod at each exposure. After a standard development of the plate the density of the central part of the rod outer limb is matched against the wedge by means of a photocell.

When the required number of exposures has been made, the retina is bleached on the slide by substituting a white light for

the monochromatic ones which have been used for photography. The whole series of exposures is then repeated on the bleached cell. In this way one can obtain a spectral absorption curve on both unbleached and bleached visual cells. It is necessary to adjust the aperture and magnification so as to minimise the effect of the outer limb envelope in scattering light. This effect is, of course, less with thicker outer limbs, and this is where *Xenopus*, which has very large rods, is a relatively easy animal to use.

Platinum Chloride Method

A retina kept in the dark and treated with 4% potash alum retains its visual purple for several days in the unbleached form (55). Similarly, if the retina of a frog is treated for at least 12 hr with platinum chloride (2 ml of $2^1/_2$% solution in distilled water introduced through the optic nerve into the bulb) after a period of dark adaptation, and is then placed in absolute alcohol for 1 hr, cleared in xylol, embedded in paraffin and sectioned, so that the rods appear in transverse section, a yellow colouration appears in a highly refractive zone around the outer segment of each rod (102). This may indicate the distribution of the visual purple in the rod itself. But it must be emphasised that distances are extremely small in a rod, and diffusion and other physicochemical processes may well alter or give a false impression of the distribution of chemical substances within the cell. This yellow colouration of the outer segments of the rods with platinum chloride, first observed by Stern (103) and subsequently used by Tansley (110), may be the basis for a recent colour reaction (8) which may apply to carotenoids more generally. Details of the method follow.

Fix the fresh eye or retina with 3 parts 2.5% platinum chloride and 1 part neutral formalin for 12–24 hr in the dark; transfer to 90% alcohol, dehydrate and embed in paraffin. Stain with Unna-Pappenheim stain (methyl green–pyronin, Grubler) for 20 min, wash with water and dehydrate in dioxane (or for 10 min in isoamyl alcohol in the presence of anhydrous sodium sulphate [20]), mount in some neutral mounting medium (not Canada balsam). Before staining, the tissue may be advantageously treated with 60% alcohol saturated with chlorine, then rinsed fully with water. By this means the staining is sharpened and the pigment bleached.

A sky-blue colouration develops in the outer segments of the rods and cones, and in some oil droplets in both the cones and the pigment epithelium. Henle's fibres, leading from the macula lutea of the primate retina, also give this colouration (93). The incidence of the sky-blue colour by this method is, however, some-

what capricious and its specificity is suspect. The colour was not found to develop in the oil droplets of the frog or chick by Francis (35). Care must also be taken that other substances, e.g., desoxyribosenucleoproteins, which have affinity for methyl green are not mistaken for carotenoids.

It is possible that the treatment with platinum chloride has some analogy with the colour reactions of carotenoids with other acid chlorides, e.g., antimony trichloride, as used in the Carr-Price test. There are no records of the detection of carotenoids in the retina by means of the standard Carr-Price reaction, in which the antimony trichloride is applied in chloroform, but when the retina of the chick is treated with antimony trichloride in the concentrated solution formed by its own deliquescence, then a transient bright blue colour appears in the oil droplets in the cones and, also, in a rather duller blue form in the oil drops in the pigment epithelium (37). The latter fades the more quickly and may well be caused by vitamin A (see p. 201).

As already noted, the differentiation of the dark- and light-staining cones in the primate retina is dependent on the fixative and is particularly noticeable in fixatives which contain mercuric chloride or trichloroacetic acid, so that it is possible that a carotenoid or carotenoid-protein complex is involved, and that "dark" cones may contain carotenoids in higher concentration than "light" cones.

FLUORESCENCE

Numerous investigators (30, 31, 44, 52, 83, 85, 89, 97, 98) have examined the fluorescence which occurs in the retina when it is illuminated with ultraviolet light. The results are of great interest and the subject might well be pursued further. Two recent investigations show how this type of method can be applied. In the first (44), the retina was removed, given a short formalin fixation, and frozen sections were cut without delay. They were examined immediately in water. Light from a mercury vapour lamp was filtered with a Corning glass filter 584 and a glass cell containing 5–10% copper sulphate. A filter in the eyepiece of the microscope cut out the ultraviolet which had passed through the specimen. It was found that methylene blue did not interfere with the fluorescence so that it could be used to stain the retina and so to localise the seat of fluorescence. A green, rather rapidly fading fluorescence seems to be characteristic of vitamin A. In the light-adapted eye this type of fluorescence occurred in certain droplets in the more peripheral parts of the pigment cells and to a less extent in the rods and cones (44). A nonfading fluorescence occurred in

the inner parts of the pigment cells, in the rods and cones, and in the outer synaptic layer, though not in the intervening nuclear layer. In the dark-adapted eye there was no fading green fluorescence, and a rust-brown fluorescence appeared where the permanent green fluorescence was seen in the light-adapted state (89). Alcohol and acetone removed the vitamin A, but dilute HCl was found to increase the fluorescence. Here again further work is needed since riboflavin, of which the eye contains an abundance, also shows a green fluorescence at certain hydrogen ion concentrations. Indeed this aspect has been studied by Sjöstrand (97, 98) in the frog and guinea pig. In the former this author records a greenish-yellow fluorescence of the rod outer segments and a bluish fluorescence of the inner segments of both rods and cones and of the outer segments of cones. The pigment layer and the choroid also have fluorescent substances, and he identifies the fluorescent substance of the choroid as riboflavin on the basis of maximum emission at 510 mμ when the substance was dissolved in acetic acid by dropping the acid onto the choroid coat.

It is possible that the histochemical method of Chévremont and Comhaire (22) for the detection of riboflavin in the liver might be successfully applied to the retina. It is unlikely, of course, that the fluorescence is of direct photochemical importance in vision, for the ultraviolet light is normally cut off before reaching the pigments. Xanthopterin and ichthyopterin are two other fluorescent substances which have been identified with the help of their fluorescent spectra in the tapetal and choroidal layers of some fishes and reptiles (83).

Valuable as these methods can undoubtedly be, great care has to be exercised in identifying substances either by fluorescence spectra or by direct absorption spectra in the cellular surroundings, since so much may depend on such factors as the pH of the medium, the presence or absence of particular proteins, the solvent and the manner in which the pigment is arranged.

POLARISED LIGHT STUDIES

It has been known since 1861 (112) that when isolated rods of the frog are examined in aqueous humour, the outer segment shows positive uniaxial birefringence, the optical axis being parallel to the length of the rod (90, 91). Alcohol reduces the birefringence and reverses its sign; presumably this depends on the action of the alcohol in disrupting the lipoid system, since the other optical qualities change at the same time. If this is so, then the lipoid molecules were orientated in a direction parallel to the length of the rod. The remaining negative birefringence is considered to be

due to form birefringence caused by a lamellar protein structure, with the lamellae running transversely to the length of the rod. This is shown by immersing the rods in media of different refractive index (water 1.33, xylol 1.49, benzyl alcohol 1.54, oil of cinnamon 1.60, α-monobromnaphthalin 1.66, methylene iodide 1.74) and finding that the birefringence is reduced to a minimum at $\mu = 1.6$. Actually it is made slightly positive again under these conditions, and this is probably due to a slight crystalline birefringence in the lamellar protein itself. The lamellar pattern is presumably to be identified with that demonstrated by the electron microscope (99, 100).

Freshly teased-out rods examined by plane-polarised light without an analyser are seen to be dichroic. If the visual purple is still present, then they appear colourless when the long axis is parallel to the plane of polarisation and purplish when at right angles to this plane. After bleaching, they are respectively colourless and yellow. Fresh rods observed in green light ($\lambda = 500$ mμ) are respectively green and black. When examined end-on, the colour of the pigment (purple or yellow) always appears, and fresh rods are black when examined with green light. Now osmic acid abolishes the positive birefringence of the lipids but leaves the dichroic character of the visual purple: presumably therefore the chromophore groups of the visual purple molecules are still orientated on the proteins in a direction parallel to the length of the rods.

For further details the reader is referred to Schmidt (90, 91) and Denton (26a, 26b).

LIPIDS

The carotenoids, which are fundamentally concerned in the photosensitivity of the eye, are fat-soluble pigments, and while they are sometimes directly visible, as in the outer segments of fresh rods, or in the oil globules in the cones of birds or in the pigment epithelium, they may also occur masked in other lipids. The nature of these lipoid materials, which abound in the retina, is therefore worthy of investigation, but the techniques for their identification leave much to be desired. The following reactions for the detection of lipids may be summarised.

Fixation, in most cases, by Baker's formal-calcium (3) is a safe beginning.

40% formalin	10 ml
10% aqueous sol of anhydrous $CaCl_2$	10 ml
Distilled water	80 ml

The solution is stored over powdered chalk. Fix the tissue for

about 6 hr, more or less, depending on size and the method of application.

1) Sudan III, for all lipids liquid at room temperature, especially triglycerides. Stain frozen sections with Sudan III in 50% ethanol for 20 min. Rinse in 50% ethanol. Counterstain with haemalum. Mount in glycerin or similar mounting medium.

2) Sudan black B, for all lipids liquid at room temperature. A saturated solution of Sudan black B is made in 70% ethanol and filtered before use. Treat frozen sections with 70% ethanol for 30 sec. Stain for 10–30 min in a closed vessel. Rinse for 30 sec in 70% ethanol, then 50%, and wash well in distilled water. Counterstain with 1% safranin and mount in glycerin.

3) Nile blue sulphate.—A rose colour (due to the oxazone) indicates neutral lipids (hydrocarbons, triglycerides and esters) provided that these are not mixed with fatty acids, lipins or chromolipids, which take on the blue colour of the oxazine. Cholesterol does not appear to stain. A general blue coloration of the tissue has no particular significance (14).

The tissue is fixed in 10% neutral formalin or Baker's formal-calcium for 24 hr, washed in running water for 6 hr, and frozen sections are prepared. The sections are placed in dilute Nile blue sulphate solution and left overnight at room temperature. Differentiation is carried out in 2% acetic acid, the sections are washed in distilled water and mounted in dilute glycerol.

To prepare the Nile blue solution (14), add 50 ml of 0.5% sulphuric acid to 500 ml of saturated aqueous solution of Nile blue sulphate; boil in a reflux condensor for 2 hr. Cool, filter the reagent and store in a warm place. The solution is filtered before each use. For use, make a 1% solution from this stock solution.

4) Baker's acid haematein test (3, 15).—This is essentially a refinement of the older Smith-Dietrich test (65), and if combined with the pyridine extraction test is specific for lipins, such as lecithin, cephalin and sphingomyelin. Special solutions required are:

a) Dichromate-calcium

Potassium dichromate	5 g
$CaCl_2$ (anhydrous)	1 g
Distilled water	100 ml

b) Gelatin for embedding

Cresol 0.25% aqueous sol	100 ml
Finest gelatin	25 g

Soak the gelatin for an hour in the cresol solution. Warm until the gelatin dissolves. Strain through muslin when still warm.

c) Acid haematein. Place 0.05 g haematoxylin in a flask.

Add 48 ml of distilled water and exactly 1 ml of 1% aqueous solution of sodium iodate. Heat until the water just begins to boil. Cool. Add 1 ml of glacial acetic acid. Use on the day of preparation. Special haematoxylin for determination of pH (British Drug Houses) is satisfactory.

d) Borax ferricyanide

Potassium ferricyanide	0.25 g
Borax (sodium tetraborate, 10 H_2O)	0.25 g
Distilled water	100 ml

e) Weak Bouin's fluid

Picric acid (satd aqueous sol)	50 ml
Formalin	10 ml
Glacial acetic acid	5 ml
Distilled water	35 ml

PROCEDURE.— *1*) Fix in formal-calcium; 6 hr. *2*) Transfer directly to dichromate-calcium; 18 hr. *3*) Transfer to oven at 60° C; 24 hr. *4*) Wash in running water; 6 hr. *5*) Transfer to melted gelatin at 37° C; 12 hr. *6*) Solidify in refrigerator; cut suitable block and leave in formal-calcium; 12 hr. *7*) Wash in running water; ½ hr. *8*) Cut sections on freezing microtome. *9*) Transfer sections to oven at 60° C in dichromate-calcium; 1 hr. *10*) Wash in several changes of water; 5 min. *11*) Acid haematein at 37° C; 5 hr. *12*) Rinse in distilled water. *13*) Borax-ferricyanide at 37° C; 18 hr. *14*) Wash in several changes of water. *15*) Mount in Farrant's medium or glycerin jelly.

Performed according to the foregoing instructions, this test stains lipins blue, blue-black or grey; carotene pale brown, all other tissues, etc., pale yellow, except for mucin and caseinogen, and a few other proteins which may show a pale greyish colour. Allowance can be made for these proteins by treating parallel tissues as follows: *1*) Fix in weak Bouin's fluid; 24 hr. *2*) 70% alcohol; 1 hr. *3*) 50% alcohol; ½ hr. *4*) Pyridine, 2 changes, each 1 hr. *5*) Pyridine at 60° C; 24 hr. *6*) Running water; 2 hr. *7*) Dichromate-calcium, and proceed as from *2*) in the acid haematein test. Only the proteins now remain positive, as the lecithin, etc., have been dissolved by the pyridine.

5) *Romieu's test for lecithin* (87). — Fix the tissue in 10% neutral formalin or formal-calcium. Dehydrate in anhydrous acetone. Embed rapidly in paraffin, with two changes of paraffin. Cut sections 5–10 μ thick and fix them to slides. Remove the paraffin with warm acetone in a closed vessel. Pass through warm water to eliminate glycogen. Place the slide in 10% hydrochloric acid and warm over a small flame until it begins to give off vapour.

Without washing, treat for several minutes with

Iodine	6 g
Potassium iodide	8 g
Distilled water	100 ml

Wash the slide for a few seconds with distilled water and mount in glycerin. Lecithin is coloured mahogany red, and the colour fades in a few hours. Other structures are coloured yellow.

6) Ciaccio's methods (65).—A. This tests for the presence of lipins and mixtures of cholesterol or cholesterides with oleic acid. Fix for 2 days or longer in

5% $K_2Cr_2O_7$	80 parts
Formalin	20 parts
Formic acid	5 drops

Transfer to 3% $K_2Cr_2O_7$ for 5–8 days. Wash in running water for 24 hr. Dehydrate and embed in paraffin. Cut sections and bring them down to 70% alcohol; stain for 1 hr at 37° C in 90 ml of 80% alcohol plus 5 ml of acetone saturated with Sudan III or Sudan black (by warming to 50° C and cooling). Wash in 50% alcohol, counterstain with haematein or safranin. Mount in Apathy's medium.

B. If the whole process is preceded by extraction in acetone, then only lipins are stained. Fix in formal-calcium and transfer to the following solutions. Keep the tissue for about 1 hr in each.

SOLUTION	1	2	3
10% Cadmium nitrate	10 ml	10 ml	10 ml
Acetone	70 "	80 "	90 "
Water	20 "	10 "	—

Then transfer to acetone containing 2% of a saturated solution of cadmium nitrate in absolute alcohol, renew several times in 24–48 hr, and warm the last bath to 37° C. Rehydrate the tissue with the acetone in reverse order, each containing 1% cadmium nitrate, and transfer to acetone plus 5% $K_2Cr_2O_7$ and gradually pass to pure 5% $K_2Cr_2O_7$ and keep it in this solution for 2 or 3 days at 37°. Cut frozen or paraffin sections and stain with Sudan III or Sudan black. In preparing the paraffin sections, dehydrate in increasing concentration of acetone; pass to acetone and petrol ether (30–50° B.P.), then to petrol ether, and on to petrol ether and paraffin at 37°, and finally to paraffin.

7) Modified Schultze-Liebermann-Burchardt test (65, 66).—This procedure detects sterols, e.g., cholesterol, etc. *a*) Fix in formal-calcium for about 36 hr. *b*) Prepare frozen sections at about 15 μ. *c*) Place the sections in 2.5% ferric alum at 37° C; 24 hr. *d*) Mount the section on a slide, and blot it carefully. *e*) Cover with several drops of a mixture of 10 ml of concentrated sulphuric acid plus

5 ml of glacial acetic acid, which has been kept for at least 2 days in a loosely stoppered bottle, during which time the reagent picks up a little water, which is necessary for the development of the full colours. *f*) Examine directly.

The pure sterols in the test tube or on cigarette paper give the following colours, so that the colour appearing in the section may result from any mixture (67):

Cholesterol	Deep green
Testosterone	Clear blue-green
Desoxycorticosterone	Brownish green
Dehydroandrosterone	Brownish green
Oestrone	Pink-mauve
Progesterone	No colour

Carotenoids also give blue colours with concentrated sulphuric acid, and galactolipines give red-purple colours. In investigating the retina these substances may well destroy the usefulness of the test, or at any rate complicate the interpretation of results. In the frog's retina, the nuclei appear red-purple, the synaptic layers green, and the rods and cones brown (37). The same colours are produced rather more quickly if hydroquinone (67) is used in place of acetic acid, in the following way: 2 g hydroquinone is dissolved by heating in 70% (v/v) conc sulphuric acid, and the mixture used on ferric alum-treated sections as with the Schultze reagent. This reaction is based on Brown's (13) modification of the Kober reaction.

8) *Windaus's digitonin test, for cholesterol* (65).—*a*) Fix in formal-calcium; 6 hr. *b*) Cut frozen sections. *c*) Place the sections for several hours in 0.5% digitonin in 50% alcohol. *d*) Rinse in 50% alcohol. *e*) Rinse in water. *f*) Mount in Apathy's medium, or glycerine jelly.

Examine under the polarising microscope. Cholesterol produces crystals and rosettes. The crystals do not stain with Sudan III, and they are not formed from cholesterol esters.

9) *Carotenoids* (see also pp. 194 and 195).—A. Antimony trichloride reaction (37). Antimony trichloride is a highly hygroscopic material and on keeping for some time it absorbs water and produces a brown viscid fluid. Fix tissues in 10% neutral formalin for 6 hr. Wash in running water for 30 min. Soak in gum arabic and cut frozen sections. Place the section on a slide and blot off the excess of water with filter paper. Apply the antimony trichloride directly to the section, cover with a coverslip and examine directly. It is essential to carry out all the procedures very rapidly, as otherwise oxidation by atmospheric air will prevent the development of the blue colour. Although demonstrating carotenoids

in some oil droplets, this test is not successful in detecting those in the outer segments of the visual cells.

B. Method of Berger and Segal (8) (see p. 194).

Up to the present, the retina has been rather unsystematically examined with regard to its lipoid content, and a more critical analysis by methods with greater specificity is much needed. Some general remarks may, however, be of interest.

Osmic acid is not a specific reagent for fats, and blackening may result from any sufficiently reducing substance.

It is vitally important that the specificity of the various reactions be considered in the light of the fixatives chosen and of any solvents used in the preparation of the tissues; the Sudan stains, for example, can be made more specific by the judicious use of suitable solvents, but the following observations indicate the necessity for caution. The Sudan stains colour the outer segments of rods and cones, and also, though less heavily, the inner segments. Both synaptic layers are also coloured. Biochemical analysis of isolated rod outer segments indicates a high phospholipin content (about 31.9%), but Baker's acid haematein test, while strongly positive on the retinal nerve fibres, is only feebly positive on rods and cones.

The outer segments of the rods are highly birefringent, a property known to be due in part to lipids, and cholesterol is perhaps the lipid which most often displays this property in biological material. Moreover, digitonin, which reacts with cholesterol, is also one of the more effective agents for extracting visual purple from the outer segments of the rods (108). Saponin also liberates visual purple and forms a complex with cholesterol. All these observations might be taken to point to the conclusion that cholesterol must be an important constituent of the outer segments of rods. It may well be important, but it is evidently not present in suf ficient amounts to give a positive reaction in the Schultze test for cholesterol, or, if it is, then the green is masked by the reaction with something else, for, apart from the optic nerve fibres, the only parts of the retina to give the green reaction characteristic of cholesterol are the synaptic layers. In these, the presence of cholesterol may account for much of the sudanophilia. Furthermore, it must be remembered that digitonin also has other actions besides that with cholesterol which might be responsible for its action in liberating visual purple; for example, it is known to have the capacity of allowing retinene to go into aqueous solution (23). Bile salts too are effective in liberating visual purple, and their action in haemolysis is thought to lie more on the proteins than on

the lipoid constituents of the cell surface, though their action on lipoid material in the alimentary canal is of course well known.

Another anomaly on somewhat similar lines is noticed in the "staining" of the colourless "oil droplets" present in some cones in the retinae of birds and frogs. These droplets, which appear to be homologous with the other coloured droplets in birds, are, like them, easily blackened by osmic acid. On the other hand, they do not, for some unknown reason, take up the Sudan dyes.

All these observations point very clearly to the fact that the histochemical localisation of particular constituents in particular cells, depending as it must on numerous isolated pieces of evidence, is a matter of great uncertainty and requires most rigorous controls.

GLYCOGEN

Best's carmine stain and iodine in potassium iodide have both been used on the retina by numerous authors (12, 68, 72, 77, 92, 107) when this has been fixed in alcohol, acetone or Carnoy's fluid. The paraboloids of those cones which possess them are either composed of glycogen or at least contain it; but the glycogen in this situation is not so labile as it is, for example, in the liver. The paraboloid itself in the cones of the frog always gives a positive reaction with Best's carmine (provided it is suitably fixed): in addition, well-fed frogs and "summer" frogs show glycogen extending centrally round the cone nucleus and into the synaptic processes. This disappears in winter and during starvation (77). The iodine reaction is not so strong as one might expect if the whole mass of the paraboloid were glycogen, and the colour is less definite than it is with liver glycogen. The colour of the paraboloids with iodine is not very different from the colour of the outer segments of the rods, where it must be due to some other substance, since Best's carmine produces no reaction in these structures. Amylase takes longer than it does in other tissues to prevent the development of such staining with iodine as occurs and to make the paraboloid negative to Best's carmine stain. Paraboloids, as discussed later, give a positive periodic-Schiff reaction, but this does not necessarily mean that glycogen is present.

NUCLEOPROTEINS

Desoxyribosenucleoproteins and ribosenucleoproteins are both stained by such dyes as Nissl's methylene blue and toluidine blue and are stained more or less specifically by Giemsa's stain, the former becoming reddish purple and the latter blue (51), and by pyronine-methyl green which stains them greenish to purple and

red respectively. This last stain is made up and used as follows (51, 117):

Pyronine G or Y (Grubler, B.D.H., or Geigy)	0.3 g
Methyl green (Grubler, or Anachemia, C. 1.685)	0.3 g
Glycerol	20 ml
Absolute ethanol	2.5 ml
0.5% Phenol in distilled water to	100 ml

The mixture is ground in a mortar, then boiled for 2 min and filtered. Stain for 20 min, wash rapidly in distilled water, and dehydrate for 10 min in isoamyl alcohol in the presence of anhydrous sodium sulphate.

By using these stains, the proportions and positions of the two types of nucleoprotein can be determined and the findings can be verified by digestion with ribonuclease or desoxyribonuclease. Crystalline ribonuclease can be obtained from the pancreas by the methods of Kunitz (56) or McDonald (70), and desoxyribonuclease by the method of McCarty (69). As prepared in this way, desoxyribonuclease should be further purified by repeated fractionation with ammonium sulphate. Since it still possesses a slight proteinase activity, the enzyme solution is made up with cysteine or hydroxylamine, which inhibits the proteinase without interfering with the desoxyribonuclease. A 0.02% solution of the enzyme in water is diluted with an equal volume of either 0.1M cysteine hydrochloride or 0.1M hydroxylamine hydrochloride in 0.1M sodium acetate at pH 6.8. The mixture is then warmed to 37° C and diluted with veronal buffer to pH 7. Solutions are unstable and should be made up for each occasion (51).

The Feulgen reaction, after mercuric chloride fixation and the usual treatment with ethanol, is fairly specific for desoxyribosenucleoproteins and stains only the nuclei in the retina.

Sections which have been fixed in methyl alcohol and stained with Unna-Pappenheim's pyronine-methyl green show Nissl's bodies very well developed in the large ganglion cells, and much more diffuse staining of the smaller ganglion cells. Changes have been observed in the distribution of Nissl's granules in the ganglion cells in the eyes of birds as the result of exposure to light (19). Similar pyronine-positive material occurs in the cytoplasm of amacrine cells, in some other cells near the outer synaptic layer which are probably the horizontal cells, and in the inner segments of cones and, to a less extent, of the rods also in some animals. The nucleolus of the cone nucleus is pyronine-positive in some animals, and this may be used as a guide in the separation between rod and cone nuclei. As mentioned earlier, frozen sections of the retina, after formalin fixation, show that the outer segments of the

rods may become heavily stained with pyronine by the Unna-Pappenheim method. Without parallel tests using the specific enzymes, this method cannot be relied upon entirely to locate the position of the two types of nucleoprotein, and it must further be remembered that ribosenucleoproteins are rather easily extracted in saline solutions.

SCHIFF REACTION

Schiff's reagent for aldehydes (fuchsin in SO_2 solution) can also be used for obtaining several types of information about retinal constituents when the tests are properly controlled.

The reagent is made up as follows: Dissolve 1 g basic fuchsin in 200 ml of boiling water cooled to 50° C, add 20 ml N · HCl, cool to 25° C and add 1 g of sodium bisulphite, and store the solution in a well-stoppered bottle. It should be a pale straw colour.

Applications.—The reagent can be used in several ways:

1) Directly, on fresh tissues, to detect the presence of existing aldehydes. This is usually negative on the retina. Previous fixation, however, particularly with formalin, makes the outer segments of the rods Schiff-positive. Hack (45) has shown that such staining is probably an artifact caused by the formalin.

2) When fresh tissues, either as frozen sections or as smears, are treated for 2 to 10 min with 1% $HgCl_2$ and then with the Schiff reagent in a closed vessel, colouration indicates the presence of an acetal bond, provided no colouration appears in sections not previously treated with $HgCl_2$. Extraction with organic solvents will determine whether the reacting substance is a lipid. Ellipsoids and outer segments are positive, and so are the synaptic layers.

3) After mercuric chloride fixation, hydrolysis of sections with N/10 HCl at 60° C for 10 min, and subsequent treatment with alcohol to remove the so-called plasmal reaction (42, 46) which may be given by ketosteroids, aldehydes of fatty acids and acetal-phosphatides, the reagent becomes specific for desoxyribosenucleoproteins.

4) Treatment of the tissue with an oxidising agent (periodic acid, potassium permanganate, chromic acid etc., or lead tetraacetate) before applying the Schiff reagent produces further substances which develop the colour of the SO_2-fuchsin. Glycogen, polysaccharides, and mucoproteins, as for instance in the matrix of connective tissue, are all positive with the periodic-Schiff test as developed by Bauer (5), McManus (71), Hotchkiss (50) and Lillie (62, 63). Clearly, therefore, this test by itself cannot give very precise information, but, when combined with suitable fixa-

tives and pre-treatment with particular solvents, it may be expected to yield more definite answers. Under certain conditions the ellipsoids and paraboloids which are found in the cones may be specifically coloured by the Schiff reagent. For example, paraffin sections of frog retina fixed in acetone or methyl alcohol when treated with the periodic-Schiff reagent show the outer segments of the rods and cones, and the paraboloids of the cones very strongly positive. The ellipsoids, however, are unstained. On the other hand, after formalin fixation and with frozen sections the ellipsoids are positive but the paraboloids are not stained (35). This would be consistent with the view, which is also supported by other evidence (p. 203), that the paraboloid is at least partly composed of glycogen, while the ellipsoids contain Schiff-positive substances which are soluble in acetone and methanol and are thus probably lipoid in character. The method is as follows:

Reagents:

a) KIO_4 0.8 g
70% Nitric acid 0.3 ml
Distilled water 100 ml
or 5% CrO_3 in water
or 0.5% $KMnO_4$ in water

b) Schiff's reagent (SO_2-fuchsin); see p. 205.

The sections, having been brought into water, are treated with the oxidising agent for 10 min; washed in distilled water, treated with SO_2-fuchsin for 15 min, rinsed in 3 changes of water saturated with SO_2 (2 min each time), and washed in running water for 5 min. They may then be counterstained lightly with haematoxylin and again washed in water, dehydrated in alcohol, cleared in xylol and mounted in balsam.

As already indicated, the positive response from the Schiff reagent as used in this way does not immediately give precise information as to the nature of the substance giving the response, but by the judicious use of fixatives, solvents and other reagents it can be made more specific. For example, it may prove to be of interest that many carotenoids are readily converted to aldehydes by oxidation with reagents like $KMnO_4$, and retinene itself, the breakdown product and precursor of visual purple, is, as already mentioned, thought to be the aldehyde of vitamin A (75). Given the right conditions, both might be detectable by the Schiff reagent.

5) A modification of the Schiff technique has been applied to the retina by Lillie (64), and he concludes that it detects the presence of substances with carbon-carbon double bonds, and this presumably would include carotenoids, if present. It depends on a pretreatment of the tissue with performic acid. This test has given

a positive reaction in the rods of the guinea pig and some other retinae; nucleoproteins are also stained by this process, but since these can be localised by other means, they need not interfere. Fixation must be in formalin or potassium bichromate, since mercuric chloride and alcohol fixatives do not preserve the positive material in the rods. The test is performed as follows:

Performic acid reagent.— To 9.2 g 90% formic acid (8 ml) add 33.7 g 30% hydrogen peroxide (31 ml) and 0.4 g concentrated sulphuric acid (0.22 ml). The temperature must be kept below 25° C. About 4.7% performic acid is formed within 2 hr, and then the solution deteriorates, so it must be made up freshly for each batch of sections.

After fixation, bring the sections into water. Oxidise with the performic reagent for 1 to 2 hr. Wash for 10 min in running water. Treat with the Schiff reagent for 10 min. Wash in 3 changes of 0.5% sodium metabisulphite for 1 or 2 min in each. Then wash in running water for 10 min, dehydrate and mount.

Lillie considers that the reacting substance in the rods is likely to be an unsaturated phospholipin, possibly a lecithin. There would seem, however, to be alternative explanations.

LOCALISATION OF ENZYMES

CHOLINESTERASE

In view of the modern concepts of synaptic transmission, the localisation of cholinesterase in relation to a cholinergic mechanism is clearly a matter of great importance, and the retina with its schematic arrangement of cells and synapses is an excellent object for study. In addition to the partly micro-biochemical approach of estimating the enzyme in frozen sections parallel to the surface of the retina controlled by corresponding records of the histological structure as used by Anfinsen for the detection of cholinesterase (1) and similarly for diphosphopyridine nucleotides (2), 2 other methods have been applied to the retina for the detection of cholinesterase. The method developed by Koelle and Friedenwald (53) yields the more specific results. Gomori's method (43) detects other esterases (see p. 210). In all these methods great difficulty always exists in interpreting how far the enzyme is actually localised in the position at which it can be made visible. Often there are time and opportunity for diffusion to occur, either of the enzyme itself or of the products of its activity, and these difficulties are generally added to those inevitably caused by the fixation or general manipulation of the tissue. For a review of this aspect of the problem, the reader is referred to the papers by Novikoff (79) and Martin and Jacoby (73) concerned with the localisation

of phosphatases. By the following method (34), as by the biochemical assay, cholinesterase has been localised in the inner synaptic layer. Unless the pH is rigorously controlled, the site of the enzyme appears to shift to the nuclei of the bipolar cell layer, but this almost certainly is an artifact. The same explanation probably applies to the finding of cholinesterase in the ganglion cells, though the question of penetration of the reagents into the ganglion cells cannot be excluded since these cells were found to be much more positive in sections than in whole preparations (47).

Procedure

REAGENTS.*

1) Glycine 1.88 g
Potassium hydroxide 1.0N 2.0 ml
Distilled water to 50.0 ml

2) 1.0M magnesium chloride solution

3) 0.1M copper sulphate solution

4) Copper glycinate

Glycine	3.75 g
Copper sulphate	2.50 g
Distilled water to	100.0 ml

5) Ammonium sulphide solution

Conc ammonium sulphide	1.0 ml
Distilled water to	50.0 ml

6) Copper sulphide, obtained by the addition of copper sulphate solution to ammonium sulphide solution. Collect precipitate by filtration.

7) Acetylthiocholine

Acetylthiocholine iodide	69.0 mg
Distilled water	3.6 ml
Copper sulphate solution	1.2 ml

The solution is centrifuged and the supernatant preserved in the cold.

8) Copperthiocholine

Acetylthiocholine iodide	29.0 mg
Distilled water	1.0 ml
Copper sulphate solution	0.6 ml

* All chemicals can be obtained from The British Drug Houses, Ltd., Graham St., London, N., except: *acetylthiocholine*—Roche Laboratories, Welwyn Garden City, Herts., England; *tris-(hydroxymethyl)-aminomethane*—Commercial Solvents, Corp., 17 E. 42nd St., New York 17, N. Y., U. S. A.

Centrifuge and filter. To the filtrate and two 1 ml washings with distilled water add 1.0 ml each of reagents *1)* and *3)*. Adjust to pH 10 with 1N potassium hydroxide; allow to stand overnight at room temperature until precipitation is complete. Collect the precipitate by centrifugation.

Solution

a) 40% sodium sulphate	9.0 ml
Distilled water	6.0 ml

b) 20% sodium sulphate solution saturated with copperthiocholine

c) 10% sodium sulphate solution saturated with copperthiocholine.

d) Distilled water, saturated with copperthiocholine.

Solution S

Copper glycinate	0.6 ml
Maleate-tris-(hydroxymethyl)-aminomethane buffer (pH 6.0)	3.6 ml
40% sodium sulphate adjusted to pH 6.0 with 0.5N NaOH	9.0 ml
Magnesium chloride solution	0.6 ml

A very small quantity of copperthiocholine (sufficient to saturate the solution) is added, and the solution well stirred and placed in the incubator at 37.5° C for 15 min. 1.2 ml of acetylthiocholine is added, the solution is filtered and used immediately.

Diisopropylfluorophosphate(DFP) solution

DFP solution (10^{-4}, 10^{-5}, 10^{-6}M)	1.5 ml
40% sodium sulphate	9.0 ml
Distilled water	4.5 ml

Buffer solution is prepared as follows (43): 29 g of maleic acid and 30.4 g of tris-(hydroxymethyl)-aminomethane are dissolved in 500 ml of water, decolourised with a few grams of charcoal and filtered. 10 ml of this stock solution and 6 ml of 0.5N NaOH, diluted to 50 ml give a buffer solution of pH 6.0. According to the amount of NaOH added, pH values from 5.08 to 8.45 can be obtained.

Technique.—The eyeball is removed immediately on the death of the animal and immersed in chilled, ice-cold salt solution of appropriate strength. Further procedures are carried out in this solution as quickly as possible. The eyeball is opened by a circular cut a little behind the sclerocorneal junction and the retina de-

tached carefully from the sclera. It is cut into small strips and transferred to solution *a*) for 5 min and then into incubation solution *S*) for periods ranging from 10 min to 2 hr at 37.5° C. A pH value of 6.0 is most satisfactory (34). Some strips are kept in DFP solutions (10^{-7}M–10^{-5}M) for 30 minutes at 37.5° before incubating with acetylthiocholine, since "true" cholinesterase is inhibited by 10^{-5}M DFP, while other nonspecific cholinesterases are inhibited by 10^{-7}M DFP.

After the appropriate incubation period, the tissues are transferred to solution *b*) for 5 min, then to solution *c*) for 1 min and finally rinsed in solution *d*). They are then placed in the dilute solution of ammonium sulphide for 30 sec, rinsed briefly in distilled water, fixed in 10% formalin saturated with copper sulphide, dehydrated through ascending grades of alcohol, cleared in cedarwood oil and embedded in paraffin. Sections are cut at 7μ and counterstained as desired, e.g., with toluidine blue. Frozen sections may also be used.

ESTERASES

Other esterases hydrolysing esters of choline and higher fatty acids can be localised in the retina (43).

Procedure

Reagents.

1) 0.1M maleate-tris-(hydroxymethyl)-aminomethane buffer of pH 7.5 (see p 209).
2) 0.1M solution of cobaltous acetate.
3) 0.02M solution of choline ester in distilled water.
4) Dilute solution of ammonium sulphide.

Incubation solution.—A stock solution is made by adding 50 ml of cobaltous acetate solution to 100 ml of the buffer and making up the volume to 500 ml with distilled water. Add 1 mg each of calcium chloride, magnesium chloride and manganese chloride. To 50 ml of this stock solution add 1 ml of choline ester (palmitoyl or myristoyl) solution, after previously warming the stock solution to 37.5° C.

Technique.—The retina is detached from the sclera, placed immediately in chilled acetone and kept in the refrigerator for 24 hr. The tissue is then placed in 2 changes of acetone at room temperature, then transferred to xylol and embedded in paraffin (paraffin oven at 58° C for $1^1/_2$ hr). Sections (7μ) are cut and brought down through xylol and alcohols to water and incubated in the incubation solution for 2–16 hr at 37.5° C. The slides are washed in tap water for 2 min, immersed in a dilute solution of

ammonium sulphide for up to 15 min, washed again under the tap, dehydrated, cleared in xylol and mounted in balsam.

The new method described by Holt and Withers (49) based on the use of 5-bromoindoxyl acetate in the presence of a mixture of potassium ferricyanide and ferrocyanide, in which the development and localisation of the dye, indigo, indicates the presence of esterases, promises to be useful in this connection and may eventually be made specific for particular esterases.

AMINE OXIDASE

Another enzyme of immediate interest with regard to neural transmission is amine oxidase, which may be concerned with the removal of adrenaline-like substances from the synapses, and which may therefore be used as a guide to indicate whether any of the synapses depend on adrenaline or nor-adrenaline for their transmission.

Procedure 1

The method of Oster and Schlossman (81), which depends on the detection of the aldehyde formed by the action of the amine oxidase, would appear to be applicable, but there are so far no reports of its successful use in this connexion on the retina. The method consists in cutting unfixed tissue on the freezing microtome, treating the sections for 24 hr at 37° C with 2% sodium bisulphite, which should bind all those substances which may give the plasmal reaction with the SO_2-fuchsin (Schiff reagent). To test the completeness of this, some sections may be treated, after the bisulphite bath, with N 10 HCl and 1% $HgCl_2$ for 6 hr, and others treated for the same time with water, and then both lots subjected to the SO_2-fuchsin. The Feulgen test and the plasmal reaction should both be negative and the tissue should be unstained.

To test for amine oxidase, the tissues are thoroughly washed after the bisulphite bath and incubated at 37° C for 24 hr with 0.5% tyramine hydrochloride in M 15 phosphate buffer solution at pH 7.2, when the sections are then treated with the SO_2-fuchsin; a blue colour should develop in any place where the amine oxidase is present. The diffusibility of the colour is one of the main drawbacks to the method; but, as already said, the amine oxidase has not been satisfactorily demonstrated in the retina by histochemical means. If sections are treated with octyl alcohol for 24 hr after the bisulphite bath, the amine oxidase is inhibited and the tissue should then remain relatively colourless with the SO_2-fuchsin. The final preparation must in each case be thoroughly washed with sulphurous acid after treatment with the SO_2-fuchsin.

Procedure 2

The following method for amine oxidase, which was designed to investigate the possibility of the presence of adrenergic cells in the retina, gives very satisfactory results on the kidney, liver and on ganglion cells in the adrenal medulla (36) but has, so far, only given negative results on the retina itself, apart from some colouration of the ganglion cells.

Reagents.

1) 0.1M phosphate buffer of pH 7.4

2) 0.1% solution of neotetrazolium (pp-diphenylene-bis-2-[3,5-diphenyl]tetrazolium chloride)

3) 0.1% solution of tyramine hydrochloride

Technique.—The retina is detached carefully and washed in phosphate buffer for about 30 min, then placed in

0.1% neotetrazolium	1 part
0.1% M phosphate buffer	1 part
0.1% tyramine solution	1 part

for 2–4 hr at 37° C. At the end of the incubation period, the retina is removed, washed in distilled water, fixed in formalin, sectioned on the freezing microtome with or without gelatin embedding, and the sections are mounted in dilute glycerol.

Controls are done by incubating with octyl alcohol for 3 hr before incubation with tyramine, and by incubating with potassium cyanide in a final concentration of 3×10^{-3}M.

Fat stains red, and this colour can be removed by treating the sections with acetone, which dissolves away the fat and takes off the red stain also. The sections are washed in distilled water and mounted in dilute glycerol. A blue colouration indicates the presence of amine oxidase.

Procedure 3

Another method which detects amine oxidase, described by Blaschko and Hellmann (8a), might also be applied to retinal tissues. It depends on the production of a brown colour when fresh tissues, if necessary sectioned on the freezing microtome at 25 μ, are incubated in 0.067M sodium phosphate buffer at pH 7.4 in the presence of tryptamine hydrochloride (1 mg/ml). 5-Hydroxytryptamine (serotonin) produces similar effects, though the coloured product is more soluble.

SUCCINIC DEHYDROGENASE

The work of Wald and his co-workers (113–115) on the synthesis of visual purple and iodopsin from retinene, and the relation of

retinene to vitamin A (75), emphasises the importance of metabolic processes in the rods and cones. Wald has shown that succinic dehydrogenase may play a significant part in the synthesis of rhodopsin from vitamin A and retinal homogenates, and it is therefore of interest to find a very high concentration of this enzyme in the ellipsoids of the visual cells, presumably associated with their essentially mitochondrial structure. The method used for locating this enzyme follows (33, 94, 96):

Procedure

Reagents.

1) 0.1M phosphate buffer of pH 7.6.

2) 0.1% solution of neotetrazolium (pp-diphenylene-bis-2-[3,5-diphenyl], tetrazolium chloride).

3) 0.2M solution of sodium succinate.

Technique.—The retina is detached carefully from the freshly removed eyeball and washed in phosphate buffer for 15 min. It is then placed in

0.1% neotetrazolium	1 part
0.1M phosphate buffer	1 part
0.2M sodium succinate	1 part
Distilled water	1 part

for 12 hr at 37° C. At the end of the incubation period, the retina is removed, rinsed briefly in distilled water, fixed overnight in 10% neutral formalin, embedded in gelatin and sections are cut at 10–15 μ on the freezing microtome. A purplish blue colouration indicates the presence of the enzyme.

Controls are done by omitting the substrate, i.e., sodium succinate, from the incubating solution, otherwise keeping the solution the same; and by using 0.02M potassium malonate as inhibitor of the enzyme; malonate is added to the incubating solution which contains succinate also.

DOPA-OXIDASE

The dopa-oxidase test is performed as follows (57):

Procedure

Solutions required are:

1) 0.3 g dopa (3,4-dihydroxyphenylalanine for Bloch's dopa reaction; Hoffmann-LaRoche) in 300 ml of distilled water. Keep in refrigerator and discard when red.

2) 11 g disodium hydrogen phosphate in 1,000 ml of distilled water.

3) 9 g potassium dihydrogen phosphate in 1,000 ml of distilled water.

Before cutting the sections, add 2 ml of *3*) and 6 ml of *2*) to 25 ml of *1*). The pH should be 7.4. Filter.

The test should be carried out on fresh material or on material fixed for a maximum of 3 hr in 5% formalin. The reaction is sensitive to pH, and all glassware should therefore be specially clean. Stain at 30–37° C, and the reaction should be stopped when the solution has turned sepia.

Becker (6) has described a method for staining in bulk (3 mm cubes) after fixation in Bouin's fluid. The tissue is washed after fixation, then treated with dopa for 30 min, the fluid renewed and the tissue incubated for 12–15 hr at 37° C. The tissue is then dehydrated, embedded in paraffin, sectioned and brought down to running water where it is washed for 1 hr before counterstaining and mounting. Becker and his co-workers (32) have also shown that tyrosinase is present in the human skin and can be demonstrated by incubating the skin with tyrosine after subjecting it to ultraviolet light. This method might well be applied to other tissues which specialise in "tyrosine metabolism," including the pigment-forming regions of the eye.

OTHER ENZYMES

Other enzymes, like the phosphatases, can be investigated by the standard methods, but great caution must be exercised in the interpretation of results (4, 40, 41, 73, 79).

Indophenol oxidase (cytochrome oxidase) is present in the ciliary epithelium (38), as can be shown by covering a piece of this tissue with 1 ml of Ringer's solution and adding an equal volume of a mixture of 0.01M dimethylparaphenylene diamine and 0.01M alpha-naphthol in Ringer's solution. The blue colour develops in the epithelium, and it is relevant to enquire whether other tissues related to the retina may show this reaction at some stage in their development.

EMBRYOLOGICAL AND COMPARATIVE METHODS

A proper understanding of the structure and function of any organ can only be obtained when that organ is seen as the culmination of a long evolutionary and developmental process. This is particularly true in the case of the eye, and both comparative and embryological studies are of the greatest value. Many of the methods described in the preceding pages are, of course, also applicable to different animals and also to embryological material, and, as in the case of the Golgi technique, often provide more information when so used.

Glücksmann (39) has provided an interesting method of analysing the development of the eye by making counts of mitotic figures and of numbers of degenerating cells as the retina develops. If such a treatment were combined with various histochemical tests, e.g., those for nucleoproteins, glycogen, fluorescent substances, and perhaps more particularly dopa-oxidase and other enzymes, the relationship and organisation of different cell groups might be better understood. Which cells degenerate, do they represent a single type which is permanently lost, and do the same cells degenerate in other types of eyes? Is there any difference in the mitotic-degenerative sequences in eyes which will become "rod"-eyes from those which can be described as "cone"-eyes or mixed eyes? These are questions to which the answers would be very informative. The dopa-oxidase is mentioned specifically because it is positive in certain cells in the retina during the early stages of development, then becomes negative. This must indicate either that pigment is formed in the epithelium once and for all, or else, that, in later life, it is constantly being handed on from cells in the choroid coat. Glücksmann (39) also describes a curious migration of pigment in the retina of the developing tadpole at the primitive optic cup stage, from the side nearest the pigment epithelium to the position which will eventually be occupied by the ganglion cells.

Another relevant observation which has not yet been fully worked out on the cellular level, and which might prove to be a useful tool, is provided by the work of M. R. Lewis (61) on the development of tadpoles in water containing 1:500,000 phenol indophenol. This causes the tadpoles to develop without pigment in both skin and eyes. A more detailed histological and cytological

study of this effect than has so far been made might provide important information with regard to the cell types affected. For example, many cells are dopa-positive but never produce melanin, and one wonders how these react to this reagent (29). Moreover, many cells can produce melanin, at least under some conditions, and yet be dopa-negative (74, 95).

The application of the full battery of histochemical methods, and of new ones still to be developed, to the study of the chemodifferentiation of the retina and of its associated embryonic structures might well be very illuminating. Tissue culture methods, which have already been used (28, 106, 109) to study the differentiation of the eye as a whole, might well be used more, together with experiments in regeneration (104, 105), in the analysis of the various cell types and in the study of their behaviour. The formation of pigment has been successfully studied in this way (28); the potentialities of the pigment cell layer in some amphibia to form a complete new retina on the one hand (104) or a new lens on the other (27, 105) are surprising observations which need to be fitted into the general pattern of organisation and development of the eye. Such methods as that of Moscona (76) for the study of differentiation might well be applied to the eye.* By this method, embryonic tissues can be broken down to their constituent cells, which can then reorganise and re-differentiate. Calcium-free media and the enzyme trypsin are the effective agents for dissociating limb-bud rudiments and for kidney and pituitary. Collagenase and hyaluronidase have also been used but are less effective. Trypsin in combination with Ca-free and Mg-free media is also effective in dissociating viable cells from heart, liver and skin (86) and could well be extended to the retina. The greater delicacy of neural tissues may, however, make the method ineffective with the retina.

Finally, the histochemical approach needs to be applied more intensively to comparative studies of the retina which have till now been far too much on the purely morphological scale on the one hand or on the purely functional scale on the other. We need to know more about the chemical relationships between the various cell groups which make up the eye, and more about the biochemical similarities and differences between corresponding tissues in different species.

Note: In the preparation of this section the author has freely made use of an unpublished article by Prof. G. Walls and Dr. G. Underwood on histological methods as applied to the retina, which was prepared as part of a wider scheme on Methods in the Physiology of Vision. In certain

* This has now been done (76a).

parts of the work he has been greatly assisted by Dr. C. M. Francis, Dr. M. C. Lobban and Dr. E. J. Denton, who have also allowed him to make use of some unpublished observations. Dr. K. Tansley and Dr. E. H. Leach have both helped by reading the manuscript and offering many suggestions. To all these the author offers his most grateful thanks, but in particular to Prof. Walls and to Dr. Francis.

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